THE ROLE OF MAST CELLS IN GASTRO-INTESTINAL NEMATODE PARASITISM OF THE GOAT

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DECLARATION

I hereby declare that:

(i) This thesis has been composed by myself

(ii) It has not been accepted in any previous application

(iii) The work described has been carried out by myself or, where jointly, this fact has been acknowledged

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ABSTRACT

Studies were conducted to investigate the *in vivo* and *in vitro* responses of caprine mast cells to challenge with gastro-intestinal nematode parasites. Initial work concentrated on the isolation and purification of a mast cell-specific neutral granule protease, termed goat mast cell protease (GMCP), from homogenates of caprine intestinal tissue. Immunological, biochemical and molecular characterisation studies localised the enzyme to caprine mast cells, confirmed its similarity to a sheep mast cell protease (SMCP), uncovered cDNA coding for a second mast cell protease (termed GMCP II) and highlighted a dual chymotrypsin and trypsin-like substrate specificity. This latter finding categorised it alongside SMCP and bovine duodenase as a member of the novel 'janus' class of dual-specific ruminant serine esterases. Polyclonal and monoclonal antibodies were raised against GMCP and an enzyme linked immunosorbent assay (ELISA) constructed which was used to determine the functional activity of mast cells. This was achieved initially in the tissues of animals undergoing experimental infections with nematodes and subsequently in *ex vivo* populations of isolated intestinal mast cells and bone marrow derived mast cells grown *in vitro*.

In vivo studies comparing yearling goats and kids with lambs undergoing primary and secondary infections with *Telodorsagia circumcincta* demonstrated that after secondary infections the goat and kid abomasal tissues retained more worms and, after both primary and secondary infections, contained considerably less GMCP than the equivalent lamb abomasal tissues. A significant mastocytosis response following repeated exposure to the parasites was also demonstrated in both species, although the secondarily infected goats and kids contained proportionately more globule leukocytes (GL) than the lambs. Blood and tissue eosinophil responses were variable, but measurements for total serum IgE demonstrated that the goats, kids and lambs developed increased mean total serum IgE levels after primary and secondary exposure to nematodes.

Caprine mast cells were also studied in isolation through *ex vivo* purification of caprine intestinal mucosal mast cells (MMC) and GL and *in vitro* growth of bone marrow marrow derived mast cells (BMMC) from haemopoietic precursor cells using recombinant ovine cytokines. Caprine and ovine BMMC cultures were characterised in terms of their cell morphology, GMCP, SMCP and acid hydrolase content during cytokine dose and time response experiments, which demonstrated the significant effects of ovine stem cell factor on BMMC proliferation and long term culture viability when used in combination with ovine interleukin-3. Biochemical and morphological studies, including the use of electron microscopy, were carried out to compare isolated goat MMC with goat and sheep BMMC as well as with isolated sheep MMC. These highlighted significant differences in the mediator content of the different cell populations with markedly less protease in caprine BMMC when compared with ovine BMMC and caprine MMC.

Goat MMC and BMMC were stimulated to release the contents of their cytoplasmic granules in the presence of synthetic secretogogues and crude nematode antigen preparations. Initial results showed that calcium ionophore A21387 produced the greatest dose-related release of mediators from caprine BMMC, indicating that the majority of the cells present in these cultures possessed a functional phenotype similar to that associated with MMC. Subsequent studies involving crude antigen preparations derived from *T. circumcincta* and *Haemonchus contortus* demonstrated that they were capable of inducing BMMC activation. This activation response could also be enhanced after passive sensitisation of the cells with IgE-containing serum or lymph preparations obtained from animals undergoing experimental exposure to parasitic nematodes.

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LIST OF ABBREVIATIONS

ADCM	Anthelmintic disrupted challenge model
β-hex	β-hexosaminidase
µg	micrograms
48/80	compound 48/80
5-HT	5-hydroxytryptamine (serotonin)
A23187	calcium ionophore A23187
anti-GMCP	antibody to GMCP
anti-SMCP	antibody to SMCP
BCA	bichinoic acid
BLT	carboxybenzoyl-L-Lysine thiobenzyl ester
BMMC	bone marrow-derived mast cell(s)
bp	base pair
BSA	bovine serum albumin
Cbz	carboxybenzoyl
CD	cluster determinant
cDNA	complementary DNA
CFC	colony forming cell
CTMC	connective tissue mast cell(s)
dA	adenosine nucleotide
DAB	3,3'-diaminobenzidine
ddH_2O	double distilled water
DFP	di-isopropyl fluorophosphate
dig	digoxigenin
DNA	deoxyribonucleic acid
dT	thymidine nucleotide
DTNB	dithionitrobenzoic acid
EDTA	ethylene diaminotetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ES	excretory/secretory antigen preparation
FCS	foetal calf serum
FITC	fluoroscein isothiocyanate
FPLC	fast protein liquid chromatography
g	gram (or gravity for centrifugation)
GIEL	gut associated intra-epithelial lymphocyte
GL	globule leukocyte
GMCP	goat mast cell protease
HBSS	Hanks' Balanced Salt Solution
HRP	horseradish peroxidase
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modification of Dulbecco's medium
iu	international units
kD	kilodaltons
L	larvae

l	litre
LNCM	mesenteric lymph node conditioned medium
LT	leukotriene
M	molar solution
ME	mercapto-ethanol
mg	milligrams
ml	millilitre
MMC	mucosal mast cell(s)
MMCP	mouse mast cell proteinase
MRI	Moredun Research Institute
MW	molecular weight
N	normal solution
NAN	nitroanilide
NC	nitrocellulose
ng	nanograms
NPE	nitrophenol ester
NRS	normal rabbit serum
O.D.	optical density
OPD	orthophenylene-diamine
Ov	ovine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PG	prostaglandin
r.p.m.	revolutions per minute
RACE	rapid amplification of cDNA ends
RE	rapid expulsion
RMCP	rat mast cell proteinase
RNA	ribonucleic acid
rOvIL-3	recombinant ovine interleukin-3
rOvSCF	recombinant ovine stem cell factor
RT	reverse transcriptase
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	standard deviation
SEM	standard error of the mean
SMCP	sheep mast cell proteinase
SP	substance P
strept-POD	streptavidin-peroxidase
TCR	T cell receptor
TEM	transmission electron micrograph
TRITC	tetra-methylrhodamine isothiocyanate
v/v	volume in volume
VCU	villus crypt unit
w/v	weight in volume
WWA	whole worm homogenate antigen preparation

CHAPTER 1

GENERAL INTRODUCTION

1.1 Gastro-intestinal nematode parasitism in goats

1.1.1 Introduction

Gastro-intestinal parasitism is an endemic problem amongst ruminant livestock throughout the world, with species of the Trichostrongylid nematode superfamily prevalent amongst sheep and goats (Urquhart, Armour, Duncan, Dunn and Jennings 1987). The effects of intestinal parasitism range from mild, asymptomatic infections caused by low parasitic burdens, to overwhelming clinical infections which may, under extreme circumstances, cause the death of the host (Holmes 1985). Increased livestock mortality is an obvious source of economic and welfare concern, but even sub-clinical infections may result in marked loss of productivity in individual animals (Barger 1985), necessitating the development of strategies to minimise levels of infection within flocks and herds.

In goats and sheep, the detrimental effects of parasitic infection on meat, milk, wool / fibre production, liveweight gain and fertility have been recognised for many years (Barger and Southcott, 1975; Lipson and Bacon-Hall, 1976; Kloosterman, Borgsteede and Eysker, 1985; Hoste and Chartier, 1993; Barger 1985). However, the majority of available epidemiological and economic data relates almost exclusively to sheep. This is despite the fact that approximately 600 million goats are currently kept (FAO/OIE/WHO survey, 1994) equalling approximately half of the total world population of sheep or cattle. The reason for this lack of information on goats, despite their large numbers, is that in contrast to the agriculturally intensive sheep industry common in many developed countries, the majority of farmed goats are found in Third World countries where they are maintained in small numbers under semi-intensive or subsistence conditions (Lloyd 1987). In all the developed countries, FAO/OIE/WHO (1985) figures show that goats constitute less than 5% of the total

small ruminant population, while in the UK alone, where approximately 84,000 goats are kept, goat numbers are well under 1% of the numbers of sheep. Nevertheless, recent surveys by the Meat and Livestock Commission suggest that goat numbers in the U.K., in common with other Western countries, are currently increasing as farmers search for further product diversification. This has led to increasingly intensive husbandry methods being applied to the production of goat fibre, milk and meat, creating similar ecological conditions for the propagation of parasitic nematode infections in goats to those previously observed in sheep (Pomroy, 1985). In addition, the requirements for diversifying output have also increased the practice of maintaining goats and sheep together on shared pasture which, as discussed in 1.1.4, has major implications for speeding the spread of anthelmintic-resistant nematode species from goats into the susceptible sheep population (Coles and Roush, 1992).

1.1.2 Prevalence and pathogenesis of common gastro-intestinal nematodes affecting small ruminants in temperate climates.

Prevalence

In the U.K., the most common clinically important nematodes encountered by sheep and goats are *Telodorsagia (Ostertagia) circumcincta*, *Trichostrongylus vitrinus* , *Trichostrongylus colubriformis, Nematodirus battus* and, in the warmer climate of southern England, *Haemonchus contortus* (Parnell, Rayski, Dunn and MacKintosh, 1954; Boag and Thomas, 1971, 1977; Thomas and Boag, 1972, 1973; Waller and Thomas, 1978; Urquhart *et al*, 1987). Economically, the abomasal parasite *T. circumcincta* is the most significant nematode parasite to affect the U.K. sheep and goat industry (Armour, Jarrett and Jennings, 1966) although *N. battus* and *T. vitrinus* may cause significant infections in the small intestines of young lambs during their first grazing season (Urquhart *et al*, 1987).

Pathogenesis

The epidemiology and pathogenesis of gastro-intestinal nematode infections in small ruminants are broadly comparable (Thomas and Boag 1973). Therefore, findings from numerous equivalent studies carried out on the pathogenesis of nematode infections in sheep may be extrapolated to the goat (Lloyd, 1987).

Severe disruption of the host's gastro-intestinal mucosa is caused by the parasites as they develop from L_3 to L_5 larvae and emerge from the tissues, as well as by inflammatory changes caused by the host's response to infection (Miller, 1984; Miller, 1987). In *T. circumcincta* infections these changes are maximal as adults emerge from the gastric glands at about day 18 after the ingestion of infective L_3, whilst in *T. vitrinus* infections, damage occurs in the intestinal mucosa at 10-12 days post infection. Disruption of the gastric gland mass by developing *T. circumcincta* larvae results in damage to epithelial cells and hydrochloric acid producing parietal cells present within the mucosa. The parietal cells are replaced with rapidly dividing, undifferentiated, non-secreting cells which eventually spread, as the larvae develop, to encompass other surrounding, non-parasitised glands (Armour *et al.*, 1966). These changes produce an increase in the abomasal pH from 2.5 to 4.0 or 7.0, which prevents the activation of pepsinogen to pepsin and also causes a loss of bacteriostatic effect within the abomasum. In addition, there may be disruption of cell junctional complexes leading to an increase in abomasal epithelial permeability to macromolecules which may, in heavy infections, result in elevated levels of pepsinogen within the plasma as well as a concomitant hypoalbuminamia due to increased losses of plasma proteins into the intestinal lumen (Holmes, 1985; MacKellar, 1993).

1.1.3 Factors influencing the development of immunity to gastro-intestinal parasites.

Studies by Barger (1985) and Riffkin (1988) have shown that in a typical herd or flock, the spread of parasitic infection is not uniformly distributed, with often a relatively small number of individuals responsible for producing the majority of parasite eggs deposited on the pasture. Post-mortem analyses also show that these animals are responsible for harbouring the largest numbers of nematodes. This suggests a role for host-derived factors in producing this irregular spread of infection which has previously been described as an 'overdispersed' population distribution (Barger 1985).

Factors involved in regulating the host's ability to resist parasitic infections are complex and are still poorly understood. Areas which have received the greatest attention include host gender, breed, age, reproductive status and nutrition as well as the presence of immunity to the parasites (Barger, 1993; Gray, 1991; Gregg, Dineen, Rothwell and Kelly, 1978; O'Sullivan and Donald, 1973; Abbot, Parkins and Holmes, 1985). In terms of the work presented in this thesis the most important factor controlling the gastro-intestinal nematode infra-population is the presence of either innate or acquired immunity to parasite infection. Innate immunity is defined as the inherent ability of the host to regulate parasite establishment, development , persistence and fecundity, whereas acquired immunity is an active response which improves with repeated exposure to the same or similar pathogens (Patterson, 1996). In ruminants maintained under intensive production systems, it is probably the development of acquired immunity which has the greatest effect on reducing parasite burdens following periods of exposure (Barger, 1985), although the presence of innate immunity may influence the establishment and fecundity of primary infections in young animals. Acquired immunity manifests itself by causing a variety of

behavioural, functional, morphological and biochemical changes in nematode parasites (see 1.2.1) ultimately resulting in the expulsion of established and incoming worms from the alimentary tract (Rothwell, 1989). Thus, as immunity develops, the host's susceptibility to infection decreases as incoming infective larvae fail to establish in the mucosa (Miller, 1984), and already established worms fail to mature and reproduce leading to a decrease in faecal egg output (Dineen and Windon, 1980; Seaton, Jackson, Smith and Angus, 1989 a and b; Gill, Gray and Watson, 1991). As full acquired immunity is achieved, both incoming larvae and adult parasites may be expelled in a rapid 'self-cure' response first described in *H. contortus*-infected sheep (Stewart, 1953) or by density-dependent losses of adult worms as seen in Trichostrongylid infections in sheep (Waller and Thomas, 1981; McClure, Emery, Wagland and Jones, 1992). Factors influencing the onset and effectiveness of the acquired immune responses to helminths include the age of the host (Colditz, Watson, Gray and Eady, 1996), host nutrition (Holmes, 1985; Blackburn, Rocha, Figueiredo, Berne, Vieira, Cacalcante and Rosa, 1991), the size and duration of the larval challenge (Seaton *et al.*, 1989 a and b), host grazing behaviour (see 1.1.4) and, under managed systems, the effects of chemotherapy (Gibson, Parfitt and Everett, 1970; Barger, 1988).

1.1.4 Comparative studies on sheep and goat responses to intestinal parasitic infections

Over the last 20 years, a number of comparative studies involving goats and sheep have been carried out to determine the kinetics of infection in the two species and to determine whether increased host resistance occurs in goats with age and exposure to challenge (Le Jambre and Royal, 1976; Anon, 1982; Brunsdon, 1983; Brunsdon, 1986; Kettle, Vlassoff, Reid and Horton, 1983; McKenna 1984; Le Jambre, 1984; Pomroy, Lambert and Betteridge, 1986; Watson and Hosking, 1989;

Woolaston, Singh, Tabunakawai, LeJambre, Banks and Barger, 1992; Jallow, McGregor, Anderson and Holmes, 1994; Huntley, Patterson, Mackellar, Jackson, Stevenson and Coop, 1995). The results obtained all indicate much larger differences between goats and sheep than expected, do not support the view that parasitic infections progress in an identical manner in the two species and may be summarised as follows (reviewed by Lloyd, 1987). Significantly higher total worm burdens are found in goats when compared to sheep following either natural or experimental challenge (LeJambre and Royal, 1976; LeJambre, 1984; Huntley *et al.*, 1995). Following natural infection at pasture, faecal egg output is generally greater in goats than in sheep, (Le Jambre and Royal, 1976; Anon., 1982; Brunsdon, 1983; Le Jambre, 1984; Pomroy *et al.*, 1986; Watson and Hosking, 1989; Jallow *et al.*, 1994; Huntley *et al.*, 1995). The development of age and experience-related resistance to nematodes, whilst well recognised in sheep, is controversial in goats, although there is some evidence to suggest that older goats may be better able to withstand the pathological effects of heavier worm burdens (Brunsdon, 1983). Finally, many anthelmintics appear to be less effective at removing worms from goats than sheep although, as will be discussed, this may be due to differences in the pharmacokinetics of anthelmintics in goats as well as the presence of increased numbers of anthelmintic-resistant strains of nematodes.

Overall, these findings demonstrate that there are major functional differences in the immune responses to nematodes between the two species, with particular controversy as to whether adult goats are able to mount an adequate acquired immune response against nematodes. Comparative studies designed to investigate this have provided inconclusive results. Several reported the absence of any acquired immunity in adult goats (Brunsdon, 1983; Kettle *et al.*, 1983; Pomroy and Charleston, 1989b; Watson and Hosking, 1989; Woolaston *et al.*, 1992), whilst others demonstrated a degree of responsiveness that is markedly inferior and later in

onset than, that shown by sheep (Pomroy *et al.*, 1986; Pomroy and Charleston, 1989a).

Several hypotheses have been put forward to account for the apparent deficiency of the adult goat's acquired immune response to nematodes, with the widely accepted view now being that since goats are selective browsers, they would normally be less exposed to parasitic challenge than equivalent grazing animals (LeJambre and Royal, 1976). As a consequence, they have not undergone natural selection for immunity to parasites and are therefore unable to respond vigorously to large scale larval challenges encountered under farmed or experimental conditions. This has been borne out by the observation that goats appear to be much more susceptible in terms of faecal egg output and total worm burdens when grazed at identical stocking rates to sheep (LeJambre, 1984). However, recent studies on the grazing behaviour exhibited by the two species (Jallow *et al.*, 1994) have also shown that goats tend to ingest significantly larger numbers of larvae when grazed on the same pasture, possibly exaggerating this effect of increased susceptibility.

1.1.5 Strategies for reducing exposure to parasites, and anthelmintic resistance

As yet, effective commercial vaccine preparations for immunising animals against gastro-intestinal nematodes have still to be developed (Miller, 1996a; Meeusen, 1996). With the exception of an irradiated larval vaccine developed against *Dictyocaulus filariae* in cattle (reviewed, Clegg and Smith, 1978) and an experimental vaccine produced from isolated intestinal antigens of *H. contortus* (Smith and Smith, 1993), the complex way in which nematodes interact with their hosts has generally prevented the exploitation of host immune mechanisms to allow protection under field conditions. Therefore, it is the use of management methods, such as controlled grazing to reduce levels of larval challenge, along with the prophylactic use of anthelmintic drugs to minimise the effects of established infections, that still form the

backbone of current parasite control strategies (Coles, 1991). However, these schemes are not without their practical difficulties, especially where current intensive farming practices and the long term survival of some infective parasitic larvae on grass make the continual provision of 'clean' pasture for grazing susceptible animals difficult (Bairden, Armour and Duncan, 1995). Also, the more recent phenomenon of nematode resistance to the effects of anthelmintic drugs has created the possibility of susceptible animals being challenged with high levels of uncontrollable nematode larvae (Coles and Roush, 1992). This may lead to many more cases of overt clinical parasitism with associated production losses and increased mortality.

Another problem associated with the widespread use of anthelmintics has been the issue of increased drug residues in the environment, including residues found in animal products. This has led to much tighter restrictions regarding the licensing of anthelmintics in different species as well as increased drug withdrawal times prior to human consumption of milk or meat products from treated animals (Donald, 1994). Increased milk withdrawal times are of particular significance in the UK goat industry where larger numbers are kept for dairy production compared to sheep. As a consequence, many farmers may find that it is uneconomic to give prophylactic anthelmintic treatment during lactation, resulting in increased nematode populations within the herd (Lloyd 1987).

After the initial isolation of a phenothiazine resistant strain of *H. contortus* from goats 40 years ago (Drudge, Leland and Wyant, 1957), reports of anthelmintic resistant strains of nematodes have been increasing rapidly for at least the past fifteen years. Recent data has demonstrated the presence of benzimidazole resistant *H. contortus*, *T. colubriformis* and *T. circumcincta* in herds in New Zealand and Australia (Kettle *et al.* 1983; McKenna 1984; Barton, Trainor, Urie, Pyman and Wolstencroft, 1985; Scherrer, Pomroy and Charleston, 1989; Badger and McKenna, 1990; McKenna, Badger, McKinlay and Taylor, 1990), whilst strains that are

additionally resistant to levamisoles, salicylanides and avermectins either individually or in combination have also been isolated in these areas (Kettle *et al.* 1983; McKenna *et al.*, 1990; Watson and Hosking, 1990). In the northern hemisphere (reviewed Borgsteede and Coles, 1994), goat farm surveys based on faecal egg count reductions and *in vitro* egg hatch assays have shown that benzimidazole resistance exists in the UK (Scott, Bairden, Holmes and McKellar 1989; Jackson, Coop, Jackson, Scott and Russel, 1992a; Jackson, Jackson, Little, Coop and Russel, 1992b) and in France (Kerboeuf and Hubert 1985), whilst the first European combined benzimidazole/avermectin resistant strain of *T. circumcincta* has been isolated from a goat herd in Scotland (Jackson *et al.* 1992a). Overall, comparative data indicates that numbers of resistant nematodes are on the increase, and that cross-host infection between goats and sheep is occurring (Coles & Roush 1992, Borgsteede and Coles, 1994).

In view of these findings, there are a number of physiological, immunological and management factors associated with goats that make them a suitable reservoir for disseminating anthelmintic resistant nematodes. As discussed above, goats mount a relatively poor immune response when compared to sheep in the face of parasitic infection. Therefore, unlike sheep, both young and adult goats may harbour large populations of egg-producing worms. As a result, goats act as a continuous source infective larvae which may establish in other species grazed on the same pasture. In response, the farmer is required to treat his or her young and adult goats repeatedly with anthelmintics which are generally administered at the dose required for equivalent sized sheep. However, work carried out on the pharmacokinetics of different levamisoles and benzimidazoles in goats have shown that they are metabolised at a faster rate than in sheep, resulting in a much shorter period during which the drug's minimal inhibitory concentration (MIC) is achieved (Galtier, Escoula, Camguilhem and Alvinerie, 1981; Hall, Ritchie and McDonell, 1981; Kettle *et al.*

1983; Gillham and Obendorf, 1985; McKenna and Watson, 1987; Bogan, Benoit and Delatour, 1987; Sangster, Rickard, Hennessy, Steel and Collins, 1991; Hennessy, Sangster, Steel and Collins, 1993). This results in the intestinal nematodes being exposed to sub-optimal anthelmintic doses which, when combined with frequently repeated dosing at intervals matching the pre-patent period of the nematode, rapidly selects for resistant adult worms (Coles 1991). The presence of anthelmintic resistant nematodes in goats, as opposed to susceptible worms that have simply not been removed due to underdosing, has also been demonstrated through transfer of apparently resistant strains to sheep where they remained unresponsive despite dosing with the same drug at the correct MIC (McKenna 1984).

To combat this, special strategies have been advocated for the dosing of goats. These measures are based on the usage of anthelmintics at experimentally determined higher recommended dosage rates for each drug type in order to achieve the required MICs. Frequency of dosing at pasture, is reduced to no more than once every 3-4 weeks in both young and adult animals, whilst ensuring that different classes of anthelmintic are rotated at least annually. In addition it has also been recommended that goats and sheep should not be grazed on the same pasture, whilst any goats bought in should be treated with a known efficacious drug, eg. avermectin, and quarantined for at least 24hrs prior to turnout to prevent pasture contamination with resistant larvae (Scott *et al* 1989; Coles and Roush 1992).

These measures, if properly co-ordinated, may have the desired effect in the interim, although there is also recent evidence to suggest that simply increasing the dose of orally administered anthelmintics given to individual goats may not be sufficient to correct the effects of underdosing (Sangster et al. 1991). This is possibly due to the increased prevalence of oesophageal groove closure in goats at the time of drenching. In any case, the use of high doses of anthelmintics in individuals contributes to increases in the risk of drug toxicity (particularly with imidazothiazole

drugs such as levamisole, morantel and pyrantel) as well as management costs and increased residues in tissues and milk.

Therefore, in the longer term, it is important to improve our understanding of the function and regulation of mucosal immune mechanisms involved in host resistance to nematodes, to maximise the possibility of producing vaccines as an effective alternative to anthelmintics (Miller, 1996a). The following section summarises current knowledge regarding acquired ruminant mucosal immune effector mechanisms, with particular reference to possible regulatory roles played by intestinal mucosal mast cells (MMC). The final section concentrates on the biology and effector functions of MMC themselves and examines recent evidence for differences in the MMC responses shown by goats and sheep infected with gastro-intestinal nematodes.

1.2 Review of the ruminant mucosal responses to intestinal nematodes

1.2.1 Introduction

From the outset, it should be noted that the majority of data from ruminant studies applies almost exclusively to sheep and cattle with very little direct information available regarding the goat. Where data on ruminants are lacking, experimental evidence obtained from equivalent studies carried out in laboratory animals and humans will be discussed.

As outlined in 1.1.3, mucosal immunity manifests itself by altering the establishment and maintenance of parasite infections within the gut. If successful, the host effector response will generally result in one or more of the following modifications to parasite behaviour (reviewed Miller, 1984; Miller, 1987, Rothwell, 1989): Retardation or hypobiosis of larval development; morphological evidence of damage to the parasite including stunting of adult worms; reduced fecundity of the

adult females; redistribution of the parasite's predilection site within the intestine and expulsion of incoming or established larvae and/or adults from the intestine.

How resistant individuals actually bring about these modifications remains largely unknown, although numerous studies have implicated roles for a variety of overlapping specific and non-specific immune response mechanisms acting against larvae or adults present in both the intestinal lumen and within the mucosal tissues (Fig. 1.1) (reviewed Befus and Bienenstock, 1982; Miller, 1984, Miller, 1987, Rothwell, 1989, Miller, 1996a). The following section describes some of these responses, investigates how they may be initiated and regulated, as well as examining evidence for their detrimental effects on gastro-intestinal nematode populations.

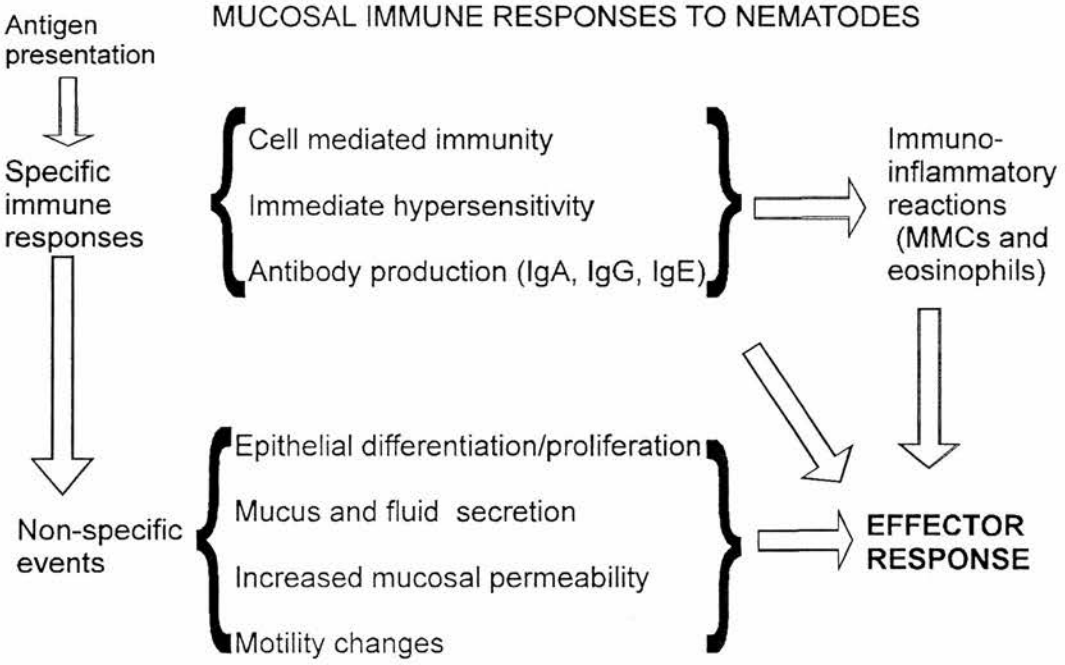


Fig. 1.1 Diagrammatic representation of specific and non-specific immune response mechanisms known to occur against intestinal nematodes (modified from Miller, 1983). See section 1.3 for more information on the role of MMC in immediate hypersensitivity reactions.

1.2.2 Antigen presentation

Information on how ruminants process nematode antigens for presentation to the immune system is currently incomplete. The induction of IgA production from B-lymphocytes in rat Peyer's patches has been demonstrated (Cebra and Shroff, 1994), therefore it is possible that dendritic cells (DCs) in gut associated lymphoid tissue (GALT) process and present nematode antigens to T cells in parasitised ruminants via the same route. This may result in the upregulation of IgA blast responses in ovine afferent gastric lymph as they develop immunity (Smith, Jackson, Jackson and Willams, 1983; Smith, Jackson, Jackson, Williams and Miller, 1984a; Smith, Jackson, Graham, Jackson and Williams, 1987). Circulating DCs have also been shown to drain from the GALT tissues in rat afferent intestinal lymph in response to the presence of endotoxin (Macpherson, Jenkins, Stein and Edwards, 1995), ending up as interdigitating cells in the mesenteric lymph node paracortex (Fossum, 1988). Here, the presence of these antigen presenting cells (APCs) within the lymph node may sensitise B-lymphocytes to induce preferential production of IgE, which is a ubiquitous feature of nematode infections (Jarrett and Miller, 1982), and has been localised to draining mesenteric lymph nodes in *N. brasiliensis* infected rats (Mayrhofer, Bazin and Gowans, 1976). It has also been hypothesised that circulating enteric DC's in ruminants possess low affinity surface receptors for the Fc components of parasite-specific IgE and IgG (Miller, 1996a) in a similar fashion to the expression of low affinity FcεRII (CD 23) receptors on human dermal DC's (Sutton and Gould, 1993). This may enable increasingly more efficient uptake and presentation of parasite allergens and antigens by DCs and B-lymphocytes within the lymph node as the immune response progresses (Harkiss, Hopkins and McConnell, 1990). This would have the effect of amplifying the mucosal antibody response, most notably of the IgE

isotype, resulting in increased mast cell activation via cross linking of parasite allergen-specific IgE bound to high affinity FcεRI receptors present on the mast cell surface (Godfrey and Gradidge, 1978; Jarrett, Mackenzie and Bennich, 1980, Metzger and Kinet, 1988). In addition, the discovery that major histocompatibility complex (MHC) class II molecules are found the surface of rat bone marrow-derived mast cells (BMMC) grown *in vitro* (see 1.3.3), and that these cells are also able to process and present immunogenic peptides to CD 4+ T cell hybridomas, suggests that intestinal mucosal mast cells (MMC) may act as APCs themselves (Frandji, Tcaczyk and Oskeritzian, 1995). Therefore, MMC may also possess an innate ability to stimulate T cell-mediated activation of humoral immune responses, including IgE synthesis (see 1.2.4; and Fig 1.2 on p 26), following exposure to parasite antigens.

1.2.3 T cell cytokine-mediated regulation of immune responses against nematodes

Unlike in murine experimental models and man (reviewed Del Prete, Maggi and Romagnani, 1994) the responses of ruminant CD4+ (in sheep, SBU $T4^+$ (Maddox, Mackay and Brandon, 1985)) T helper (Th) cell subsets have yet to be segregated into the functional categories of Th1 and Th2 on the basis of the cytokines they secrete. Despite this, the overall importance of Th cell-mediated responses in regulating ovine immunity to nematodes has now been demonstrated by the detection of increased numbers of CD4+ T cells in the lamina propria of sheep immune to *T. colubriformis* (McClure *et al.*, 1992; Kambara and Macfarlane, 1996) as well as by rendering *H. contortus* resistant sheep susceptible to challenge by antibody-mediated depletion of ovine CD4+ but not CD8+ cells (Gill, Watson and Brandon, 1993a). In the goat, the presence of distinct T cell subsets has yet to be defined, however cross reactivity with monoclonal antibodies to bovine lymphocyte antigens has demonstrated the presence of $CD2^+$, $CD4^+$, $CD8^+$ and γ/δ T-cell subsets (Navarro, Caro, Seva, Rosillo, Gomez and Gallego, 1996).

Studies examining the T cell responses of mice experimentally infected with *Nippostrongylus brasiliensis,* where the induction of mast cell, eosinophil and IgE responses typical of ruminant helminth infections occur (Miller, 1984), has shown that the type II (Th2) response predominates in these animals (Finkelman, Pearce, Urban and Sher, 1991; Finkelman and Urban, 1992; reviewed Urban, Fayer, Sullivan, Goldhill, Shea-Donohue, Madden, Morris, Katona, Gause, Ruff, Mansfield and Finkelman, 1996; Finkelman, Shea-Donohue, Goldhill, Sullivan, Morris, Madden, Gause and Urban, 1997). This results in the expression of high levels of IL-4, IL-5, IL-6, IL-9 and IL-10, in the absence of IL-2 or IFN-γ which are generally associated with type I (Th1) responses to viruses and other intracellular pathogens (Sher, Gazzinelli, Oswald, Clerice, Kullberg, Pearce, Berzofsky, Mossman, James, Morse and Shearer, 1992). It has also been demonstrated in similar experiments that during this Th2 response, it is the production of IL-4 which appears to upregulate the production of other Th2 cytokines (Fernandez-Botran, Sanders, Mossman and Vitetta, 1988; reviewed Del Prete *et al.* 1994; Urban *et al.*, 1996). Meanwhile, cytokine depletion experiments carried out in sheep have demonstrated a role for the Th1-associated cytokine IFN-γ in retarding the development of naturally acquired immunity to *T. colubriformis,* probably through the downregulation of Th2 cytokine expression (McClure, Davey, Emery, Colditz and Lloyd, 1996). These findings suggest that the pivotal role for IFN-γ in influencing Th1:Th2 cytokine ratios in parasitised laboratory animals (Gajewski and Fitch, 1988) is also of significance during ovine infections and underlines the probable importance of Th2 responses in the development of ruminant mucosal immunity to nematodes.

In laboratory animals, the host protective effects of IL-4 in particular have recently been demonstrated in mice infected with *Trichinella muris* and *Heligmosomoides polygyrus* (reviewed Finkelman *et al.*, 1997). Experiments involving the administration of anti-IL-4 or anti-anti-IL-4R monoclonal antibodies

(mAb) block host immunity to a challenge *H. polygyrus* infection (Urban, Katona, Paul and Finkelman, 1991). Similarly, treatment with anti-IL-4R mAb (which in addition to blocking IL-4 also blocks the effects of IL-13 which produces synergistic effects with IL-4 in the upregulation of IgE isotype switching, see 1.2.4 below) causes chronic *T. muris* infections in normally resistant mice (Finkelman *et al.*, 1997). The importance of IL-4 in controlling these infections has also been demonstrated in experiments in which mice with chronic infections were treated with a formulation of IL-4 that has a long half-life *in vivo* (IL-4 complexes (IL-4c) prepared by mixing IL-4 and a neutralizing anti-IL-4 mAb at a 2:1 molar ration, so that the antibody acts as a carrier protein that protects the cytokine from degradation and secretion (Finkelman, Madden, Morris, Holmes, Boiani, Katona and Maliszewski, 1993)). Treatment with IL-4c cures even established primary *T. muris* and *H, polygyrus* infections (Else, Finkelman, Maliszewski and Grencis, 1994; Urban, Maliszewski, Madden, Katona and Finkelman, 1995) whilst treatment also terminates chronic *N. brasiliensis* infections that develop in anti-$CD4^+$ mAb treated or SCID mice but not normal mice (Urban et al., 1995). This latter finding indicates that IL-4 stimulates host protective mechanisms that are normally redundant in *N. brasiliensis* infections but become critical when other CD4+ T cell-dependent mechanisms are blocked. Reports of increased numbers of muscle larvae in IgE-depleted, *T. spiralis*-infected rats and of transfer of rapid expulsion with purified IgE antibody (Harari, Russell and Castro, 1987; Ahmad, Wang and Bell, 1991) also support a protective role for IL-4, because nematode infection–induced IgE production is IL-4 dependent (Finkelman, Katona, Urban, Snapper, Ohara and Paul, 1986; see 1.2.4 below). Established effects of IL-4 that may influence worm expulsion include stimulation of IgE responses (Finkelman et al., 1986; see 1.2.4 below), stimulation of mucosal mastocytosis (Finkelman et al., 1993; Madden, Urban, Ziltener, Schrader, Finkelman and Katona, 1991; see 1.3.3 below), further promotion of Th2 cytokine responses (see above), stimulation of T

cell growth (Spits, Yssel, Takebe, Arai, Yokota, Lee, Arai, Banchereau and de Vries, 1987) and enhancement of VCAM-1 expression, (the endothelial cell receptor for the integrin VLA-4, which is involved in the migration of macrophages, lymphocytes and eosinophils across venous high endothelium) (Elices, Osborn, Takada, Crouse, Luhowskyj, Hemler and Lobb, 1990; Schleimer, Sterbinsky, Kaiser, Bickel, Klunk, Tomioka, Newman, Lucinskas, Gimbrone, McIntyre and Bochner, 1992).

Recent findings also suggest that Th cells alone do not retain a monopoly on cytokine production in infected mucosa, and many regulatory cytokines including IFN-γ, IFN-α, TGF-β, IL-4, IL-5, IL-6, IL-10 and IL-13 are also produced by $CD4^-$ T cells, natural killer (NK) cells, macrophages and mast cells (see table 1.1) (Mond and Brundswick, 1987; Plaut, Pierce, Watson, Hanley-Hyde, Nordan and Paul, 1989; Burd, Rogers, Gordon, Martin, Jayaraman, Wilson, Dvorak, Galli and Dorf, 1989; Galli, Gordon and Wershil, 1992; Ramsay, Husband, Ramshaw, Bao, Matthaei, Koehler and Kopf, 1994; Burd, Thompson, Max and Mills, 1995). Therefore, these cells may also play a significant role in either suppressing or augmenting Th2 cytokine responses to helminths. In particular, the finding that mast cells produce significant amounts of IL-4 protein in response to IgE mediated activation by allergens (see table 1.1 and Fig. 1.2, page 28) (Burd P.R, 1989; Galli *et al.* 1992) suggests that intestinal mast cells may have a major role in initiating and amplifying Th2-mediated mucosal immune responses during nematode infections (Del Prete *et al.*, 1994).

Cytokine	Evidence for mast cell-derived mRNA or bioactivity*	Function**
Interleukin-1 (IL-1) [a]	Burd *et al.*, 1989	Activation of T cells
IL-3 [a]	Burd *et al.*, 1989	Stimulates proliferation of haemopoietic precursor cells including other mast cells
IL-4 [a]	Plaut *et al.*, 1989	↑IgE production by B cells,induction of Th2 cell phenotype, ↑trans-endothelial migration of eosinophils.
IL-5 [b]	Burd *et al.*, 1989 Plaut *et al.*, 1989	Promotes growth, adhesion,transendothelial migration,chemotaxis, activation and survival of eosinophils.↑ IgA synthesis
IL-6 [a]	Burd *et al.*, 1989 Plaut *et al.*, 1989	↑IgE synthesis by B cells,promotes growth, differentiation and activation of T cells. ↑ IgA synthesis
IL-13 [b]	Burd *et al.*, 1995	↑IgE synthesis by B cells.
Granulocyte macrophage colony stimulating factor (GM-CSF) [a]	Burd *et al.*, 1989	Promotes development/differentiation of granulocyte/macrophage (GM) progenitor cells
Chemokines [a] eg macrophage inflammatory proteins (MIPs), monocyte chemotactic proteins (MCPs),eosinophil chemotactic factors e.g. ECF-A and TCA3	Burd *et al.*, 1989 Galli, Wershil, Gordon and Martin,1989	Promotes chemotaxis of inflammatory Leukocytes
Transforming growth factor-β (TGF-β) [b]	Gordon and Galli,1994	Fibroblast growth factor, chemoattractant for mast cells. modulates anti-apoptotic effect of stem cell factor (SCF) on mast cells in peripheral tissues (see 1.3.3). IgA isotype switching
Tumour necrosis factor-alpha (TNF-α) [a]	Young, Liu, Butler, Cohn, and Galli, 1987	Enhances cytotoxicity, promotes histamine and protease release from mast cells, promotes transendothelial migration of leukocytes

Table 1.1 Summary of selected cytokines and chemokines known to be produced by mast cells. * first citation of their production by mast cells (nb. data from human, rodent and murine cell sources only). ** selected effector functions which might have implications for mucosal immunity to parasites (see text). [a] Protein demonstrated, [b] mRNA only demonstrated

1.2.4 Immunoglobulin responses

Three immunoglobulin isotypes have received the most attention in terms of research into humoral responses towards nematode parasites (Miller, 1996a). These are IgA, IgG and IgE. In terms of mast cell responses to nematodes, IgE is probably the most significant (Jarrett & Miller, 1982; Metzger and Kinet, 1988).

Host immunoglobulins within the mucosa and intestinal lumen have been intensively studied for many years to elucidate their effector role against nematode antigens processed by APCs within the mucosa and local lymph nodes (Sarles and Taliaferro, 1936; Mulligan, Urquhart, Jennings and Neilson, 1965; Ogilvie and Jones, 1968; Neilson, 1969; Miller, 1980a; reviewed Miller, 1984). Overall, IgA has probably received the greatest attention due to its ability to migrate transepithelially into the lumen of the intestine (Brandtzaeg, 1981; Cebra and Shroff; 1994). Work carried out by Smith, Jackson, Jackson and Williams (1985) indicated that the mucosal IgA response is important for determining the onset of age-immunity in young lambs, and that immunity to *H. contortus* and *T. circumcincta* can be transferred to histocompatible naive sheep by immune lymphocytes in a process which involves the transfer of IgA memory (Smith, Jackson, Jackson, Williams, Willasden and Fehilly, 1984b; Smith, Jackson, Jackson, Graham, Williams, Willasden and Fehilly, 1986). Further studies by Gill, Gray, Watson, and Husband (1993b) and Gill, Husband, Watson and Gray (1994) also demonstrated that IgA levels are increased in sheep selectively bred for nematode resistance. Meanwhile, studies on unselected adult Blackface sheep have shown that a negative correlation exists between abomasal IgA concentrations, adult female *T. circumcincta* worm lengths and egg production, but that a positive correlation exists between IgA responses and inhibition of L_4 larvae (Stear, Bishop, Doligalska, Duncan, Holmes, Irvine, McCririe, McKellar, Sinski and Murray, 1995). Despite these findings, the exact anti-parasitic function of IgA in parasitized ruminants is not known, although strong negative correlations observed between intestinal IgA levels and worm length suggest that mucosal IgA may interfere with the feeding processes of the parasites (Smith, 1988).

The work of Gill *et al.* (1993b) also indicated that sheep selectively bred for resistance to *H. contortus* demonstrated elevated levels of IgG_1 subclass antibodies. The mechanisms by which immunity to parasites is conferred by this

antibody isotype are again largely unknown, although IgG_1-mediated entrapment of worms in intestinal-mucus has been noted in immune rats (Appleton, Schain and McGregor, 1988) (see 1.2.5). This mechanism has not yet been observed in ruminants, but the translocation of IgG into the gastro-intestinal tract during parasitic infections has been demonstrated in sheep (Yakoob, Holmes and Armour, 1983).

Although until recently, there were no reagents available for the specific measurement of ruminant IgE, elevated serum levels of both parasite-specific and non-specific IgE during nematode infections of rodents and man have been described on numerous occasions (reviewed Jarrett and Miller, 1982). IgE production from B cells is determined by the relative concentrations of cytokines, predominantly produced by Th cells, and by surface contact between the B cells and other cells within their microenvironment via a number of adhesion molecules and receptors (reviewed Ishizaka, 1989; Zanders, Harris, Buckham and Quint, 1992). These include the Th cell receptor CD4 (when the B-cell itself acts as an APC presenting MHC class II bound antigen to Th cells), CD40L and CD23 (FcεRII). Of particular interest are interactions between B-cells expressing CD40 and cells expressing the receptor ligand CD40L which has been shown to induce strong proliferation of human B cell populations *in vitro,* and in the presence of the correct cytokine milieu to promote IgE isotype switching, resulting in the production of IgE secreting plasma cells (Gascan, Gauchat, Aversa, van Vlasselaer and de Vries, 1991; Sutton and Gould 1993; Banchereau, Bazan, Blanchard, Briere, Galizzi, van-Kooten, Liu, Rousset and Saeland, 1994). Isotype switching from IgG to IgE in these cells is upregulated in the presence of IL-4, with a synergistic effect provided by IL-5 and IL-13, and down regulated in the presence of IL-2 and IFN-γ (Zanders *et al.*, 1992; Purkerson and Isakson, 1992). As discussed in 1.2.3, this cytokine profile probably occurs during Th2 responses occuring in intestinal nematode infections. Evidence from human studies also suggests that CD40L is expressed on a number of cell types other than T

cells, including freshly isolated mast cells and basophils, which are also able to induce the production of IgE directly (Gauchat, Henchoz, Mazzei, Aubry, Brunner, Blasey, Life, Talabot, Flores-Romo and Thompson, 1993). This indicates that mast cells, with their ability to produce Th2-like cytokines including IL-4, IL-5 and IL-13 (Table. 1.1), may play a key role in regulating IgE production independently of T cells. This mast cell-mediated autocrine regulation of IgE synthesis, in conjunction with mast cell influences on Th cell responses discussed in 1.2.3, is possibly important for amplifying local IgE concentrations (see Fig. 1.2, page 28) within the ruminant intestinal mucosa during intestinal nematode infections (Miller, 1984; Rothwell, 1989; Huntley, Newlands, Jackson and Miller, 1992b) and may thereby also promote the development of MMC-mediated type I hypersensitivity responses (Miller, 1996 a and b).

In addition to the putative role for mast cells in both promoting and executing the IgE-mediated responses, there is also evidence that the presence of IgE alone in the absence of an MMC response may be sufficient for promoting immunity to certain parasites (Bell, 1996). This hypothesis has been suggested by data from studies on *Schistosoma mansoni* infections in both humans and rats and *T. spiralis* infections in rats which demonstrated high correlations between host resistance to the parasites and host serum IgE titres (Dunne, Butterwoth and Fulford, 1992; Verwaerde, Joseph, Capron, Pierce and Dammoneville, 1987; Wang, Korenaga, Greenwood and Bell, 1990). This resistance could also be passively transferred by either administering large amounts of monoclonal rat IgE antibody to *S. mansoni* to infected recipients (Verwaede *et al.*, 1987) or, in susceptible rats infected with *T. spiralis*, by administering physiological doses of anti-*T. spiralis* IgE purified from serum after an initial 'priming' dose of purified 'Th2-like' thoracic duct $CD4^+$ $CD45RC^-$ lymphocytes from *T.spiralis* infected donor animals (Wang et al. 1990). In the passive transfer experiments, host protection could also achieved in the apparent

absence of an intestinal mastocytosis suggesting that it is the presence of parasite-specific IgE itself that is critical for the development of immunity rather than any effector function attributable to MMCs (Bell, 1996). In this respect, IgE has also been implicated *in vitro* in antibody-mediated cellular cytotoxicity reactions against nematodes present in host tissues via the activation of macrophages and eosinophils which, like mast cells, also possess surface Fcε RI and FcεRII receptors respectively (Sutton and Gould, 1993). *In vivo* IgE is also involved in the generation of Cl^- dependent increases in intestinal secretory mechanisms which act to 'flush' the intestine (Baird and O'Malley, 1987) and may also be involved in other changes to intestinal physiology resulting in the altered transit times and co-ordination of normal gut peristalsis evident in parasitised hosts (reviewed Miller, 1984). Furthermore, it may also have a more direct effect on the parasites themselves after secretion at high concentrations into the intestinal lumen (Bell, 1996).

1.2.5 Goblet cells, mucus and physiological responses

One of the first manifestations of immunity to nematode parasites shown by laboratory animals and ruminants is the rapid exclusion of increasing numbers of incoming infective larvae from the intestinal mucosa (McCoy, 1940; Russell and Castro, 1979; Cheijina and Sewell, 1974; Miller, Huntley and Wallace, 1981; reviewed Miller, 1984 ; Miller, 1987; Rothwell 1989). In experiments where immune sheep are infected with *H. contortus* or *T. circumcincta* L_3 changes to enteric epithelial and mucus-producing goblet cells contribute to this reduction in larval establishment in a process termed 'immune exclusion' (Miller, Jackson, Newlands and Appleyard, 1983).

Hyperplasia of goblet cells occurs in parasitised sheep (Newlands, Miller and Jackson, 1990). Studies have also shown that such upregulation is probably immune-mediated and that mucus is important for reducing larval establishment since the

protective response against *H. contortus* L_3 is lost in immune sheep pre-treated with mucolytics or corticosteroids (Miller and Huntley, 1982). Earlier *in vitro* studies by Lee and Ogilvie (1981 and 1982) showed that rat intestinal mucus contains both non-specific and specific heat-sensitive components that promote the exclusion of larvae. These were subsequently shown to consist of complement and IgG_1 antibodies that are able to bind to *T. spiralis* larvae in passively immunised rats. In combination, these components create conditions which are favourable for increased physical trapping of larvae within the mucus layer (Appleton *et al.*, 1988). Altered gut physiological mechanisms have also been demonstrated in rodents, including intestinal smooth muscle hyperplasia (Castro and Harari, 1982) and active secretion of chloride ions into the intestinal lumen by epithelial cells (Harari *et al.*, 1987; Baird and O'Malley, 1993). Both contribute to increased intestinal motility and fluidity, thus minimising the chances of successful larval attachment to the mucosa.. Findings by Australian workers suggest that the above mechanisms also occur in parasitized sheep (McClure *et al*, 1992; Jones, Emery, McClure and Wagland, 1994) augmenting previous studies demonstrating the presence of 5,6 - leukotrienes (LTC_4, LTD_4 and LTE_4) in sheep gastro-intestinal (GI) mucus that inhibit larval motility (Douch, Harrison, Buchanan and Greer, 1983). LTC_4 is found in ovine BMMC grown *in vitro* (see 1.3.3) (Huntley, 1991), whilst work using isolated sheep mast cells and *ex vivo* intestinal loops from parasitised and control animals has demonstrated that ovine MMC are an important souce of leukotriene-mediated anti-parasitic activity *in vivo* (Douch, Morum and Rabel, 1996; Pernthaner, Cabaj, Shaw. Rabel, Shirer, Stankiewicz, and Douch, 1996). Sheep mast cell protease (SMCP), which is found in ovine MMC (see 1.3.5 below), has also been detected in increased amounts in mucus from sheep infected with *H. contortus* (Huntley *et al.*, 1992b), *T circumcincta* (Stevenson, Huntley, Smith and Jones, 1994) or *T. colubriformis* (Jones *et al.*, 1994; Pernthaner *et al.*, 1996). Elevations in mucus SMCP concentrations coincide with

reductions in faecal egg counts from animals undergoing primary challenge, implying that release of SMCP into intestinal mucus is associated with the onset of immunity (Jones *et al.*, 1994).

1.2.6 Eosinophils

Elevated numbers of eosinophils in the blood and mucosal tissues of rodents, ruminants and humans infected with gastro-intestinal nematodes have been noted in many studies (reviewed Befus and Bienenstock, 1982; Miller, 1984; Rothwell, 1989; Buddle, Jowett, Green, Douch and Risdon, 1992; Jones, 1993). Comparable increases in tissue eosinophil numbers have also been described in goats and sheep infected with *T. circumcincta* and *T. vitrinus* (Huntley *et al.*, 1995).

Despite the positive relationship between eosinophil numbers and parasite infection, their role against nematodes within mucosal tissues remains to be established due to conflicting results in the studies carried out to date (reviewed Rothwell, 1989, Miller, 1984; Butterworth 1984; Miller, 1996a). However, the presence of high individual blood eosinophil counts in lambs has been associated with resistance and the suppression of faecal egg counts (Buddle *et al.*, 1992 ; Kimambo, MacCrae, Walker, Watt and Coop, 1988).

In terms of the regulation of eosinophil responses, *in vitro* and *in vivo* studies in rodents and *in vitro* studies in humans have shown that, like mast cells, eosinophil proliferation, differentiation and recruitment is controlled by Th2 cytokines (Korenaga and Tada, 1994). These include IL-5 and IL-3 in the bone marrow which promote selective eosinopoiesis from haemopoietic stem cells (Saito, Hatake, Dvorak, Leiferman, Donnenberg, Arai, Ishizaka and Ishizaka, 1989; Dvorak, Saito, Estrella, Kissell, Arai and Ishizaka, 1989) and the local production of IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) within the lamina propria of the intestine. These cytokines are thought to promote eosinophil

recruitment, proliferation, differentiation and survival within the mucosa in response to the presence of intestinal parasites (Korenaga and Tada, 1994). IL-5 is currently the best characterised of the locally produced cytokines, playing a significant role during eosinophil recruitment in murine tissues *in vivo* (Sher, Coffman, Hieny, and Cheever, 1990; Hom and Estridge, 1994). MMC have been implicated as an important source of this cytokine in mucosal tissues (Plaut *et al.*, 1989; Bradding, Feather, Wilson, Bardin, Heusser, Holgate and Howarth, 1993) (Table. 1.1). Similar mechanisms may be operating in the parasitised intestine to those described in the asthmatic lung (reviewed Kroegel, Virchow, Luttmann, Walker and Warner, 1994; Warner and Kroegel, 1994). Here, allergen-mediated mast cell activation appears to drive eosinophil recruitment via the production of IL-5, IL-4 and TNF-α, eosinophil chemotactic tetra-peptide factors (ECFs), such as ECF-A (Table. 1.1), and 'newly synthesised' arachidonic acid metabolites including leukotrienes, platelet activating factor (PAF) and prostaglandin D_2 (PGD_2) (see 1.3.4 and Fig 1.2). In addition to these cytokines and peptides, a particularly potent eosinophilotactic factor has also been described (Griffiths-Johnson, Collins, Rossi, Jose and Williams, 1993). Termed eotaxin, this member of the C-C or beta subfamily of monocyte chemoattractant proteins has been isolated and characterised in guinea-pigs, mice, humans and rats, where it has generally been localised to epithelial and vascular endothelial cells in the lung (reviewed, Luster and Rothenburg, 1997). Eotaxin has yet to be described in ruminants, and its production by mast cells has also yet to be shown in any species. However, recent experiments involving the use of normal and genetically mast cell mast cell deficient (see 1.3.3 below) W/W^V mice have shown a delay the onset and reduction in the total numbers of eosinophils present in the peritoneum of W/W^V mice but not normal mice following the intra-peritoneal administration of recombinant murine eotaxin (Harris, Komater, Marett, Wilcox and Bell, 1997). This suggests that mast cells may play a role in amplifying eotaxin-induced tissue eosinophilia, although

the mechanisms involved, failing the direct production of eotaxin by the mast cells themselves or the synergistic effect of other mast cell-derived mediators, are currently unknown.

In summary, there are a number of different physiological, cellular and humoral mechanisms which are thought to influence the course of nematode infections *in vivo*. The interactions occurring between these effector mechanisms are becoming more evident as the inter and intra-cellular signalling pathways involved in regulating mucosal immunity are dissected, and it is interesting to note the prominent role played by MMC in this respect, with their particularly rich content of pharmacologically active mediators, chemokines and cytokines (reviewed Schwartz and Austen, 1984; Schwartz, 1994; Gordon, Burd and Galli, 1990, see table 1.1 and Fig. 1.2). If successful vaccines to nematodes are to be produced, an approach might be to manipulate MMC responses in an attempt to mimic the mechanisms which occur during natural immunity. Whether these responses can be influenced by immunisation with natural or recombinant antigens remains unknown and a fuller understanding of these processes is therefore required.

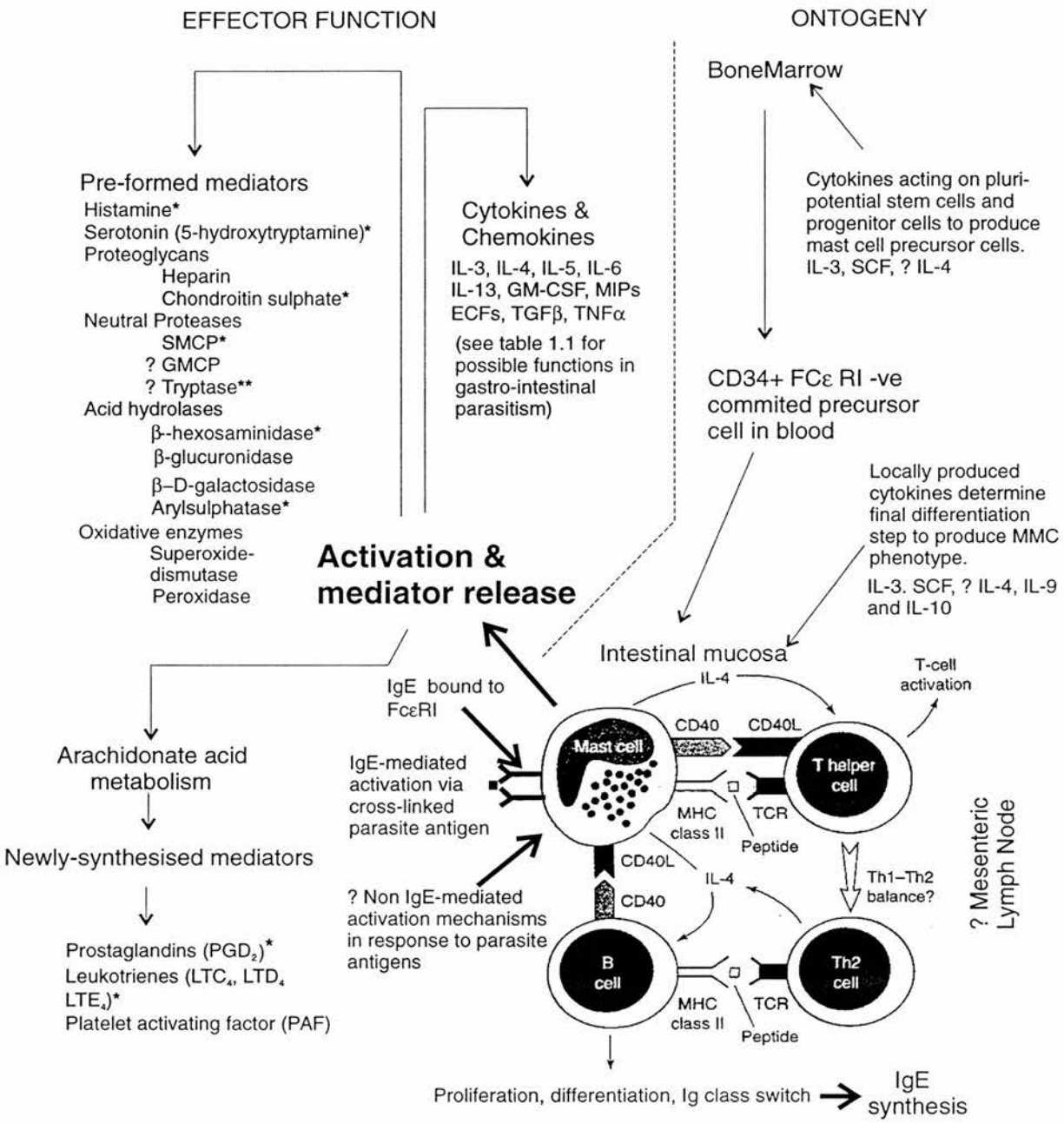


Fig 1.2 Summary of possible interactions between mast cells, T cells and B cells for the upregulation of IgE production as well as possible cytokine influences on the ontogeny and the development of MMC within intestinal tissues. Mast cell-derived cytokines and mediators released in response to cell activation are also summarised (see text; data from Schwartz and Austen, 1984, Schwartz, 1994). * Pre-formed and newly-synthesised mediators that have been identified in isolated sheep MMC/GL and mesenteric lymph node conditioned medium (LNCM) stimulated sheep BMMC (Huntley, 1991; Huntley et al., 1992; Bendixsen et al., 1995). **Immunohistochemical staining for tryptase has been observed in ovine MMCs using a polyclonal anti-human mast cell tryptase antibody (J. Huntley, H.R.P. Miller, personal communication)

1.3 Functional biology of mucosal mast cells and globule leukocytes and their role in the ruminant immune response against nematodes

1.3.1 Introduction

A key feature of the developing host immune response to nematodes involves the induction of IgE-mediated type I hypersensitivity reactions within the gut mucosa (Jarrett and Miller, 1982, Fig.1.1). These reactions may be responsible for promoting the expulsion of nematodes by a variety of mechanisms (reviewed Miller, 1996b) including : the release of low molecular weight inflammatory mediators which directly affect worm survival from allergen activated cells (Rothwell, 1989; Douch *et al.*, 1996); an IgE-mediated epithelial chloride ion secretory response which helps to flush the parasites from the mucosal surface (Harari, Russell and Castro, 1988; Baird and O'Malley, 1993, see 1.2.4 above); and increased mucosal permeability during gut anaphylaxis, promoting the pathotopic transfer of immunoglobulins and antigens across the epithelial barrier (Scudamore, Thornton, McMillan, Newlands and Miller, 1995a). This final effect may help to ensure the optimal 'priming' of APC dependent immune effector mechanisms outlined in the previous section by increasing their exposure to parasite-derived antigens.

The cells responsible for mediating such reactions are likely to be the MMC, since they possess FcεRI receptors necessary for the binding of parasite specific IgE, they the release of a wide variety of mediators following cross-linking of surface bound IgE by parasite antigens, and they also usually increase markedly in number in intestinal tissues during parasitic infection (Jarrett and Miller, 1982; Miller, 1984; Huntley, 1992). Intestinal mastocytosis has been triggered experimentally by infecting both laboratory animals and ruminants with helminths. These include *N. brasiliensis* and *T. spiralis* infections in rats and mice (Taliaferro and Sarles, 1939; Miller and Jarrett, 1971; Ruitenberg and Elgersma, 1976) and *T. colubrifirmis*, *H. contortus* and

T. circumcincta infections in sheep (Murray, Miller and Jarrett, 1968; O'Sullivan and Donald, 1973; Gregg *et al.*, 1978). The use of highly sensitive immunoassays for mast cell-specific granule-associated neutral proteinases have demonstrated that MMC are functionally active and release these mediators at the time of nematode expulsion from the immune host. This activity was originally demonstrated in rats infected with *N. brasiliensis*, and *T. spiralis* (Woodbury, Miller, Huntley, Newlands, Palliser, Wakelin, 1984) but has also been described in mice infected with *T. spiralis* (Huntley, Gooden, Newlands, MacKellar, Lammas, Wakelin, Tuohy, Woodbury and Miller, 1990) and sheep infected with *T. circumcincta* (Huntley, Gibson, Brown, Smith, Jackson and Miller, 1987).

In the following section, the origin, development and intrinsic functions of MMC in response to intestinal nematode infections will be described. The discussion will also examine a further population of intra-epithelial cells called globule-leukocytes (GL), which may be derived from MMC in the sheep (Huntley, Newlands and Miller, 1984a) and are usually found in increased numbers in parasitised ruminants (reviewed Askpavie and Pirie, 1989, Huntley, 1992).

1.3.2 *The origin and heterogen*eityof mast cells

Origin

Mast cells are derived from pluripotential haemopoietic stem cells which originate in the bone marrow (Crowle and Reed, 1984; Kitamura, Shimada, Hatanaka and Miyano, 1977; Haig, 1993) and migrate via the bloodstream as immature CD 34+, FcεRI- committed progenitor cells (Kitamura, Matsuda and Hatanaka, 1979; Metcalfe, Mekori, Rottem, 1995) (Fig. 1.2). They complete their differentiation into mature FcεRI+ve mast cells in peripheral tissue sites (Kitamura *et al.* 1979). As will be discussed further below, full cell differentiation involves development into a functional mast cell phenotype within the destination tissue. This latter step appears to

be largely dependent on relative cytokine concentrations present within the local tissue micro-environment (Kitamura, Kanakura, Sonoda, Asai and Nakano, 1987; Gurish, Pear, Stevens, Scott, Sokol, Ghildyal, Webster, Hu, Austen and Baltimore, 1995). In sheep, increased generation of multipotential eosinophil, macrophage and mast cell/basophil colony forming cells (CFCs) in bone marrow has been demonstrated within 10 days of primary or challenge infections with *T. circumcincta.* This indicates that an increase in myelopoiesis, including the production of ruminant mast cell precursors, occurs in response to nematode infections (Haig, Stevenson, Thomson, Percival and Smith, 1995)

Heterogeneity

Mast cells in peripheral tissues exhibit metachromasia when stained with thiazine dyes and are present within the tissues of a wide variety of vertebrate species (reviewed Riley, 1959; Selye, 1965; Huntley, 1992). In ruminants, their tissue distribution has been described in cattle (Kim and Lee, 1985) and sheep (Chen, Alley, Manktelow and Davey, 1990; Sture, Huntley, Mackellar and Miller, 1995), whilst studies carried out in the goat demonstrated their presence in biliary (Rahko, 1972 a and b) and gastro-intestinal tissues (Huntley *et al.*, 1995). As a cell population, they exhibit considerable structural, biochemical and functional heterogeneity which appears to be dependent on the species and tissue location under investigation (reviewed Huntley, 1992). For example, early studies in rats utilising different tissue fixatives and staining with thiazine or copper pthalocyanine histochemical dyes demonstrated different sub-populations of mast cells present in the gut mucosa and the connective tissues of the rat dermis (Enerback, 1966 a and b). After staining with an alcian blue/safranin sequence the cytoplasmic granules of MMC stained blue whereas the majority of dermal connective tissue mast cells (CTMC) stained red (Enerback, 1966b). This effect was found to be due to differences in the degree of

sulphation of intragranule glycosaminoglycans present within the cells (reviewed Enerback, 1986), and provided the first evidence that MMC possess distinct phenotypic characteristics. Studies into rat mast cell heterogeneity were extended by biochemical analyses of mast cell granule constituents (reviewed Huntley, 1992), when it was demonstrated that heterogeneity also occurs in the distribution of two isolated intra-granule neutral proteases. These were termed rat mast cell protease I (RMCP-I) and rat mast cell protease II (RMCP-II) (Woodbury, Gruzenski and Lagunoff, 1978; Woodbury and Neurath, 1980). Differences in antigenicity between the two proteases enabled the production of specific antibodies against each (Gibson and Miller, 1986), and using dual immunofluorescence, it was demonstrated that rat MMC and CTMC represent two approximately separate populations of mast cells containing RMCP-II and RMCP-I respectively (Gibson, Mackellar, Newlands and Miller, 1987). Further studies showing MMC and CTMC granule protease heterogeneity have now been carried out in other species including mice (Newlands, Gibson, Knox, Grencis, Wakelin and Miller, 1987; Le Trong, Newlands, Miller, Charbonneau, Neurath and Woodbury, 1989, Newlands, Knox, Pirie-Shepherd and Miller, 1993), humans (Miller and Schwartz, 1989; Irani and Schwartz, 1994) and dogs (Schechter, Slavin, Fetter, Lazarus and Fraki, 1988; Myles, Halliwell, Ballauf and Miller, 1995) (see chapter 4), whilst recent work in sheep (Sture *et al.* 1995) indicated that mast cell heterogeneity may also occur in this species, based on the distribution of a sheep mast cell granule protease (SMCP). In this latter study, it was found that the majority of intestinal MMC stained positive for SMCP, whilst only a small percentage of dermal and hepatic mast cells contained the enzyme. This indicates the presence of a possible ovine CTMC subset in these tissues. Equivalent studies have yet to be carried out in goats, although Huntley *et al.* (1995) demonstrated that goat MMC stain positive with antibodies to SMCP, indicating that antigenic cross reactivity occurs between SMCP and a putative intestinal goat mast

cell protease. Recent studies carried out on mast cells in human respiratory tissues have also demonstrated the presence of mast cell heterogeneity on the basis of their cytokine content (Bradding, Okayama, Howarth, Church and Holgate, 1995). This suggests that the biologic functions of mast cell sub-populations may also differ as a result of their capacity to generate and release different cytokine profiles, although these findings have not been extended to other species.

Functional heterogeneity of mast cell sub-populations has been defined in both *in vitro* and *in vivo* derived mast cells from a number of species through their responses to chemical secretogogues (reviewed Barrett and Pearce, 1993). This effect was initially demonstrated *in vivo* in the rat by injecting the polycationic secretogogue Compund 48/80 which depleted peritoneal CTMC but not MMC of their mediators (Enerback, 1966c). The neuropeptide substance P (sP) has been used to distinguish MMC from CTMC in a variety of species (reviewed Regoli, Bondon and Fanchere, 1994) and it activates human cutaneous CTMC but not intestinal MMC via a cell-receptor dependent pathway (Church, Benyon, Rees, Lowman, Campbell, Robinson and Holgate, 1989). In contrast, calcium ionophore A 21387 acts by non-specifically increasing intracellular calcium levels in a variety of cell types, activating calcium-dependent cytoplasmic granule release mechanisms. Its effects are not entirely restricted to mast cells, and it has been shown to activate both CTMC and MMC populations present in laboratory animals (Pearce, 1986) and man (Benyon, Lowman and Church, 1987; Cohan, Massey, Gittlen, Charlesworth, Warner, Kagey-Sobotka and Lichtenstein, 1989). Currently, there is no data on the existence of functional heterogeneity in goat mast cells, although studies carried out *in vitro* on ovine BMMC grown cells in the presence of mesenteric lymph node conditioned medium (LNCM) or recombinant ovine interleukin-3 (rOvIL-3) (see 1.3.3) have shown that they are activated in the presence of A 21387 (Huntley *et al*, 1992a; Sture, 1996). Moreover, ovine BMMC grown in rOvIL-3 are also relatively refractory to

stimulation with compound 48/80 or sP (Sture, 1996). This, in combination with the fact that the majority of these cells contain SMCP, (Huntley *et al.*, 1992a, Sture, 1996) indicates that they may possess an MMC phenotype, making them a potentially useful tool for investigating ruminant intestinal MMC responses *in vitro*.

1.3.3 The development of MMC in vivo and in vitro

MMC hyperplasia is a T cell-dependent process (Jarrett and Miller, 1982 ; Miller, 1996b). This was demonstrated by the finding that athymic or T cell depleted rats or mice as well as human AIDS patients have reduced numbers of mast cells in their mucosal tissues (Mayerhofer and Fisher, 1979; Ruitenberg and Elgersema, 1976; Irani and Schwartz, 1989). In parasitised hosts, experiments involving the adoptive transfer of immune rat mesenteric lymph node cells or T-cell enriched thoracic duct lymphocytes produce a mastocytosis in *N. brasiliensis* infected recipients (Nawa and Miller, 1979) whilst experiments utilising antibodies to the Th2 associated cytokines IL-3 and IL-4 suppress helminth-induced mastocytosis by up to 90% in mice infected with *N. brasiliensis* (Madden et al., 1991). In sheep, the administration of immunosuppressive doses of glucocorticoids abolishes MMC hyperplasia in *H. contortus* infected animals (Huntley *et al.*, 1992b).

As discussed in 1.2.3 murine and human studies have now shown that it is the production of Th2 cytokines which promote characteristic host responses to nematode infections including MMC hyperplasia, upregulation of IgE production and recruitment of eosinophils to the intestine (Del Prete *et al.*, 1994). This correlates with earlier *in vitro* experiments demonstrating that T cell-derived cytokines, including IL-3 (Ihle, 1985), are responsible for the *in vitro* growth and differentiation of rat BMMC which exhibit the phenotypical characteristics of enteric MMC (Haig, McKee, Jarrett, Woodbury and Miller, 1982; Haig, McMenamin, Redmond, Brown, Young, Cohen and Hapel 1988a). However, similar experiments carried out in mice

have shown that murine BMMC grown in the presence of IL-3 alone (Razin, Ihle, Seldin, Mencia-Huerta, Katz, LeBlanc, Hein, Caulfield and Austen, 1984) do not fully differentiate into MMC-like cells since they do not contain mouse mast cell proteinase-1 (MMCP-1), an enzyme found in all murine enteric MMC cytoplasmic granules *in vivo* (Miller, Huntley, Newlands and Irvine, 1990). To induce these cells to express this proteinase *in vitro* also requires IL-4 and IL-10 (Ghildyal, McNeil, Stetchshulte, Austen, Silberstein, Gurish, Sommerville and Stevens, 1992), whilst IL-9 induces the accumulation of mMCP-1 mRNA transcripts (Eklund, Ghildyal, Austen, Stevens, 1993). This indicates that full control of MMC development and phenotypic expression in this and possibly other species, is likely to be under the control of at least four Th2 derived cytokines, namely IL-3, IL-4, IL-9 and IL-10 (reviewed Sutton and Gould, 1993; Del Prete *et al.* 1994; Rennick, Hunte, Holland and Thompson-Snipes, 1995).

A non T cell-derived cytokine which has also received a lot of attention in terms of mast cell development is stem cell factor (SCF), also known as the c-kit ligand because it binds to c-kit tyrosine kinase receptors (Zsebo, Williams, Geissler *et al.*, 1990b). C-kit is expressed on the surface of 1-5% of CD34+ haemopoietic precursor cells within the bone marrow, in certain human leukaemic blood cell lines, and on the surface of both immature and mature mast cells (Galli, Zsebo and Geissler, 1994). The importance of SCF in regulating mast cell ontogeny and differentiation was first recognised when it was found that Sl/Sl^{d} and W/W^{v} strains of inbred mice, exhibiting spontaneous mutations at the loci responsible for SCF and c-kit receptor expression respectively, exhibited a profound reduction in their numbers of tissue mast cells (Kitamura, Go and Hatanaka, 1978; Kitamura and Go, 1979; reviewed Galli *et al.* 1994). SCF has now been cloned and expressed from a variety of species including mice, rats, humans and sheep (Galli *et al.* 1994; C. McInnes, personal communication) and is expressed as a transmembrane polypeptide on the surface of

murine liver stromal cells (Zsebo, Wypych, McNiece *et al.*, 1990a), murine and human bone marrow stromal cells (McNiece, Langley and Zsebo, 1991; Aye, Hashemi, LeClair, Zeibdawi, Trudal, Halpenny, Fuller and Cheng, 1992), rat thymic stromal cells (Williams, Bertoncello, Kavnoudias, Zsebo and McNiece, 1992), murine 3T3 fibroblasts (Zsebo *et al.*, 1990b) and human vascular endothelial cells (Aye *et al.*, 1992). Soluble SCF is generated either by alternative splicing of mRNA transcripts or from the membrane bound form by proteolytic cleavage (Galli *et al.*, 1994). In experiments carried out *in vivo* in rodents, the intravenous administration of recombinant rat SCF for a period of two weeks doubled the numbers of rat enteric MMC whilst the administration of anti-SCF antibodies profoundly reduced the MMC population in normal rats (Newlands, Miller, Mackellar and Galli, 1995) and mice (Grencis, Else, Huntley and Nishikawa, 1993), 1993). *In vitro* experiments involving rat BMMC cultures demonstrated that SCF alone could stimulate and maintain the production of rat mast cells although it also caused a concomitant reduction in the granule content of the MMC-associated chymase RMCP-II when compared with cells generated using IL-3 or LNCM (Haig, Huntley, MacKellar, Newlands, Inglis, Sangha, Cohen, Hapel, Galli and Miller, 1994). Furthermore, the addition of SCF to cultures enhances IgE dependent mediator release from rat BMMC (Hill, Macdonald, Thornton, Newlands, Galli and Miller, 1996). This indicates that SCF has an important role in regulating the growth, differentiation and activation of MMC in rats and that it acts to maintain MMC numbers within peripheral tissues of both rats and mice. In humans, the presence of SCF, as shown either from the addition of recombinant SCF or by co-culture with fibroblasts expressing SCF, is required for the generation of mast cells from bone marrow cells, peripheral blood mononuclear cells or umbilical cord haemopoietic precursor cells (Valent, Sanblochl, Sperr, Sillaber, Zsebo, Agis, Strobl, Geissler, Bettelheim and Lechner, 1992; Durand, Migliaccio, Yee, Eddleman, Huima-Byron, Migliaccio,

Adamson, 1994) and also enhances IgE-dependent mediator release (Valent *et al.*, 1992). Unlike rodents and ruminants, human cultures stimulated with IL-3 alone produce basophils instead of mast cells (Kirschenbaum, Goff, Dreskin, Irani, Schwartz and Metcalfe, 1989). I*n vitro* experiments have shown that once human mast cell precursors target to peripheral tissues, their survival may be dependent upon local production of SCF, since withdrawal or antibody-mediated neutralisation of the cytokine results in mast cell apoptosis (Metcalfe *et al.*, 1995).

In ruminants, studies on the development of mast cell populations *in vivo* and *in vitro* have been hampered by the lack of recombinant cytokine reagents. As in the rat (Haig *et al*, 1982), early *in vitro* studies using ovine bone marrow cells demonstrated that mast cells could be grown in cultures stimulated with LNCM from mitogen-activated mesenteric lymph node cells harvested from nematode-infected sheep (Haig, Blackie, Huntley, Mackellar and Smith, 1988b). Although described as being morphologically immature, these cells contain the MMC-like neutral granule protease SMCP and possess functional and biochemical characteristics analogous to those found in MMC isolated from the intestines of parasitised sheep (Huntley *et al.*, 1992a). More recently, the gene for ovine IL-3 has been cloned and expressed (McInnes, Haig and Logan, 1993) and initial studies have been carried out which demonstrate that ovine BMMC can be generated in the presence of rOvIL-3 alone (Sture, 1996). These cells also appear immature in terms of morphology and mediator content when compared with isolated ovine MMC, although, as discussed in 1.3.2, they are functionally active, demonstrating characteristics analogous to those found in isolated MMC (Sture, 1996). As yet, no studies have been carried out to assess the effects of recombinant ovine SCF (rOvSCF) on ovine bone marrow cultures either alone or in combination with ovine IL-3, nor any experiments done to determine whether these ovine cytokines influence the development and phenotype of mast cells in goat bone marrow cultures. These two questions will be addressed during the

course of these studies in order to provide populations of mast cells for analysing the effects of nematode antigens on the functional activity of ruminant MMC *in vitro*.

1.3.4 Mucosal mast cell mediators and the role of neutral granule proteinases in intestinal hypersensitivity reactions

Isolated sheep MMC release their mediators following parasite antigen cross-linking of surface-bound antigen specific IgE (Jones, Huntley and Emery, 1992). In addition, activation of MMC has been recorded in IgE-deficient mice (Oettgen, Martin, Wynshaw-Boris, Deng, Drazen and Leder, 1994), and in the apparent absence of an IgE response during the expulsion of *T. spiralis* infections in rats (Miller, Woodbury, Huntley and Newlands, 1983) indicating that activation of MMC may also occur through other, non-IgE mediated mechanism (Miller, 1996a). These may include, antigen-mediated cross-linking of surface bound IgG (Ishizaka and Ishizaka, 1984; Katz, Raizman, Gartner, Scott, Benson, Austen, 1992), the release of antigen-specific T cell-derived factors (Askenase, Rosenstein and Ptak, 1983), and histamine releasing factors (HRFs) from a variety of other cell types (reviewed MacDonald, 1996), as well as the surface binding of complement fragments including C3a and C5a (Pearce, 1989; Mousli, Hugli, Landry and Bronner, 1992). Selected secretogogues and neuropeptides produced *in vivo* have also been implicated in non Ig-mediated MMC activation including somatostatin, vasoactive intestinal peptide (VIP) and neurotensin (Barrett and Pearce, 1993; Selbekk, 1983; reviewed Tam, 1995). Recent reports also suggesting that these neuropeptides may mediate their effects via direct contact between MMC and intestinal nerves (reviewed Tam, 1995). In addition, there is evidence for mast cell mediators themselves being able to upregulate mast cell responses with RMCP II able to activate mast cells *in vitro* (Schick, Austen and Schwartz, 1984; Schick and Austen,1986) whilst other non mast cell-derived allergenic proteases such as bee venom allergen phospholipase A2 also cause IgE-independent activation in a process that requires functional activity of the

enzyme (Dudler, Machado, Kolbe, Annand, Rhodes, Gelb, Koelsch, Suter and Helm, 1995). Finally, studies on the *in vitro* development of mast cells have also demonstrated the release of mast cell mediators in the apparent absence of immunoglobulins, secretogogues or antigen, indicating a regulatory role for constitutive and induced release of mediators following exposure to cytokines and growth factors in the tissue micro-environment (see section 1.3.3). Most notably these include the ability of SCF to cause direct mediator release in rat BMMC and rat peritoneal mast cells (Haig et al., 1994; Taylor, Galli and Coleman, 1995) as well as MMCP-1 release from murine BMMC exposed to IL-9 and TGF-β (H.R.P. Miller, personal communication). The importance of IL-9 and the activation of mouse MMC in parasite infections has also been demonstrated *in vivo* during *T. spiralis* infections in mice. In these experiments, accelerated worm expulsion seen after the administration of recombinant murine IL-9 was prevented by the administration of antibodies to c-kit (see section 1.3.3) which block the development of mucosal mastocytosis (Grencis *et al.*, 1993, reviewed Finkelman et al., 1997).

The recognition that non-Ig specific mechanisms are capable of causing mediator release from mast cells has increased recent interest in their role during the initiation and development of innate responses which may occur on initial exposure to parasites or bacteria (Galli and Wershil, 1996; Mecheri and David, 1997). In this respect, innate mediator release from mast cells, including their ability to generate cytokines, may have a direct effect on pathogens as well as being able able to influence the development of subsequent acquired specific immune responses through Th2 cytokine-mediated IgE isotype switching (see section 1.2.4). This innate ability has been characterised most recently in experiments which demonstrate that the release of pre-formed mast cell-derived TNFα is critical *in vivo* for promoting the clearance of virulent strains of *Klebsiella pneumoniae* from the lungs and peritoneal cavities of mice (Malaviya, Ikeda, Ross and Abraham, 1996) and reduces mortality in

a mouse model of induced septic peritonitis (Echtenacher, Mannel and Hultner, 1996). Work is now currently under way to determine whether similar innate responses can be induced after exposing mast cells to nematode allergens and antigens both *in vivo* and *in vitro*.

Whichever mechanism is employed, MMC activation usually results in the release of a range of 'pre-formed' and 'newly-synthesised' mediator substances into the local tissue micro-environment (see Fig. 1.2, page 28) (reviewed Schwartz and Austen, 1984; Schwartz, 1994) as well as a variety of cytokines and chemokines which may promote the intestinal allergic-immune responses through the upregulation of IgE production and recruitment of pro-inflammatory cells including further MMC and eosinophils (Table 1.1) (reviewed Gordon *et al.*, 1990; Bradding, 1996).

Pre-formed mediators are generally associated with the mast cell cytoplasmic granules where they are held in the quiescent cell by ionic interactions with a negatively charged heparin or chondroitin-sulphate proteoglycan matrix . These include biogenic amines such as histamine, 5 hydroxytryptamine (serotonin, 5-HT) and dopamine; acid hydrolases such as β-hexosaminidase and arylsulphatase, and the mast cell-specific neutral granule proteases (Fig. 1.2). Newly-synthesised mediators, on the other hand, are produced at the time of cell activation from cell membrane phospholipids, processed by either the cyclo-oxygenase or lipoxygenase pathways of arachidonic acid metabolism to produce prostaglandins (PGD_2) and thromboxanes (Tx) or leukotrienes (LTB_4, LTC_4, and LTD_4) respectively (Fig. 1.2).

In ruminants, elevated serum and intestinal histamine levels have been recorded in a number of *in vivo* experiments involving sheep infected with intestinal nematodes (Stewart 1953 ; Jones, Windon, Steel and Outteridge, 1990; Jones *et al.*, 1994). Experiments involving isolated MMC as well as *in vitro* derived ovine BMMC have demonstrated that these cells also contain histamine, serotonin (5-HT) and dopamine (Bendixsen *et al.*, 1995; Huntley *et al.*, 1992a) and, in the case of the

isolated cells, are able to release histamine on exposure of the cells to larval antigens (Bendixsen *et al.*, 1995). The precise effects of these biogenic amines on nematodes in the intestine are unknown, although experiments carried out in guinea pigs infected with *T. colubriformis* have implicated histamine in the expulsion of adult worms in this species (reviewed Rothwell, 1989). Experiments involving the use of lambs challenged with *T. colubriformis* also showed negative relationships between GI mucus histamine levels, adult worm fecundity and larval motility (Jones *et al*, 1990; Jones *et al.*, 1994). As discussed in 1.2.5, newly synthesised eicosanoid mediators possess a direct anti-parasitic activity by inhibiting larval migration in GI mucus and also promote the recruitment of eosinophils. Like histamine, the production of leukotrienes from isolated ovine MMC and GL has been stimulated *in vitro* using *T. colubriformis* and *H. contortus* larval antigens (Bendixsen et. al. 1995), implicating these cells as a major source of the mediators *in vivo*.

As outlined in the introduction to this section, the most convincing evidence that mast cells are functionally active during nematode infections has come from immunoassay techniques measuring mast cell neutral granule protease concentrations in tissues, serum, gastric lymph and GI mucus (reviewed Miller, 1996b). These proteases (proteolytic enzymes) are classified as serine esterases due to the presence of a serine residue in the catalytic site and also because they possess a characteristic 'catalytic-triad' of histidine^{57}, aspartate^{102} and serine^{195} residues positioned around the substrate binding pocket (Springman and Serafin, 1995). Classically, these enzymes have been divided into groups of chymotrypsin-like ('chymase') or trypsin-like ('tryptase') enzymes depending on their substrate specificities (Hartley, 1960). Mast cell chymase enzymes have been described in man, rat, mouse, dog and sheep (Schechter, Fraki, Geesin and Lazarus, 1983; Woodbury, Everitt and Neurath, 1981; Le Trong et al. 1989; Caughey, Viro, Lazarus and Nadel, 1988; Huntley, Gibson, Knox and Miller 1986) whilst mast cell tryptases are found in humans, dogs, rats,

mice and cattle (Schwartz, Lewis and Austen, 1981; Caughey, Viro, Ramachondran, Lazarus, Borson and Nadel, 1987; Reynolds, Stevens, Lane, Carr, Austen and Serafin, 1990; Kido, Yokogoshi and Katanuma, 1988; Shikimi and Kobayashi, 1986). In humans, active mast cell tryptase is a tetramer comprised of four 32-34 kilodaltons (kD) subunits which are inactive when dissociated (Schwartz, 1994), whereas human and sheep mast cell chymases are active as 30 kD and 28kD monomers respectively (Schechter, 1995; Miller, Huntley and Newlands, 1995). In terms of substrate specificity, tryptases cleave peptides with basic/positively charged lysine or arginine residues at the P_1 (C -terminal) substrate cleavage site (Schwartz, 1985; Schwartz, 1994) whilst chymases cleave peptides with large hydrophobic residues such as tyrosine, phenylalanine and leucine at the P1 site (Powers, Tanaka, Harper, Minematsu *et al.*, 1985).

Sheep mast cell chymase (SMCP) was first isolated from ovine abomasal MMC (Huntley et. *al.*, 1986) and was originally classified as a serine endopeptidase enzyme with chymotrypsin-like activity on the basis of its substrate specificity (Knox, Gibson and Huntley, 1986; Knox and Huntley, 1987). Antibodies raised against the enzyme demonstrated its immunohistochemical localisation to mast cells in the ovine gut as well as other tissues (Huntley *et al.*, 1986; Sture *et al.* 1995) and has also allowed the construction of an ELISA for quantifying SMCP concentrations in intestinal tissue samples (Huntley *et al.*, 1987). These antibodies also show cross-reactivity with a putative enzyme present in goat MMC and GL (Huntley *et al.*, 1995) and a principal objective of this study will be to isolate and characterise such a caprine protease as a potential marker for mast cell activation in goats. More recent characterisation carried out on SMCP (Pemberton, Huntley and Miller, 1997a; Pemberton, Belham, Huntley, Plevin and Miller, 1997b) involving NH_2-terminal amino acid sequence analysis of peptide substrate cleavage products has shown that, in addition to its chymotrypsin-like properties, SMCP possesses catalytic activity

normally associated with tryptases. This property has been seen in another ruminant intestinal protease recently isolated from bovine duodenal mucosa termed bovine duodenase (Zamolodchikova, Vorotyntseva and Antonov, 1995a; Zamolodchikova, Vorotyntseva, Nazimov and Grishina, 1995b). Comparisons of the known amino acid sequences of these two proteases indicate that duodenase is likely to be the bovine equivalent of SMCP (Pemberton *et al.* 1997a). As a result, they have now been recognised as members of a new class of dual-specific ruminant chymases analogous to the 'janus-faced' human cathepsin G protease, which is able to cleave substrates with either phenylalanine or lysine residues at their P1 site. This is possibly due to the presence of a positively charged glutamate^{226} residue which divides the base of the enzyme's substrate binding pocket (Hof, Mayr, Huber, Korzus, Potempa, Travis, Powers and Bode, 1996). In the course of these studies, it will be interesting to determine whether the putative goat mast cell protease also shares these unusual catalytic properties.

Apart from their usefulness as markers for mast cell activity, the biological function of mast cell proteases is currently under intensive research to elucidate their roles in a number of physiological and pathological states (reviewed Caughey, 1995). However, in terms of the gastro-intestinal response to nematodes, recent work carried out in rats has demonstrated a potentially important role by causing increased mucosal epithelial permeability following their release from MMC granules. Using an *ex vivo* perfusion system of the mesenteric vasculature and jejunal lumen it was shown that anaphylactic release of RMCP-II in response to administration of *N. brasiliensis* antigens results in a significant increase in intestinal epithelial permeability to macromolecules (Scudamore *et al.* 1995a). In addition, direct infusion of RMCP-II into the mesenteric artery results in increased mucosal permeability to albumin within three minutes of infusion but does not produce any significant histological lesions within the epithelium (Scudamore, Pennington, Thornton, McMillan, Newlands and

Miller, 1995b). This implies that in addition to previous hypotheses which suggested a role for mast cell proteases in causing non-specific disruption to the Type IV collagen anchoring the epithelial cells to the intestinal basement membrane (Patrick, Dunn, Buret, Miller, Huntley, Gibson and Gall, 1988), RMCP-II is also responsible for altering epithelial permeability via a paracellular route by substrate-specific proteolysis of epithelial junction complex proteins (Scudamore et. al., 1995b; reviewed Miller, 1996a). These findings may explain why it is advantageous to have increased numbers of MMC in close contact with the epithelium in the parasitized mucosa, where they may play a role as 'epithelial-gatekeepers' allowing the pathotopic transfer of plasma proteins (including antibodies), inflammatory mediators and worm antigens to and from the gut lumen (Miller, 1996a).

1.3.4 Globule leukocytes; their derivation from mucosal mast cells

Globule leukocytes (GL), with their characteristic acidophilic staining intra-cytoplasmic granules or globules, are found within the intestinal mucosal epithelium in a variety of species including rats (Taliaferro and Sarles, 1939; Ruitenberg and Elgersma, 1979), mice (Crandall, Crandall and Franco, 1974), humans (Gustowska, Ruitenberg, Elgersma and Kockiecka, 1983), cattle (Miller, Murray and Jarrett, 1967; Murray, Miller and Jarrett, 1968); sheep (Sommerville, 1956; Miller *et al.*, 1967, Murray *et al.* 1968) and goats (Huntley *et al.*, 1995). They are particularly prominent in laboratory animals and ruminants infected with intestinal nematodes (Taliaferro and Sarles, 1939; Sommerville, 1956; reviewed Gregory, 1979) especially goats (Huntley *et al.*, 1995). The origin of these cells has been controversial (reviewed Askpavie and Pirie, 1989; Huntley, 1992) with some workers maintaining that they are derived either from modified plasma cells (Dobson, 1966), or lymphocytes (Kent, 1966) and not from mast cells (Whur, 1966). However, early ultrastructural and histochemical studies (Jarrett *et al.*, 1967; Miller *et al.*, 1968) suggested that GL may be end-stage

cells resulting from MMC degranulation since intermediate transitional cell (TC) stages were detected between degranulating MMC and GL in rats, sheep and cattle. This hypothesis was later strengthened by work carried out on mast cell deficient W/W^V mice infected with *T. spiralis* where the proliferation of GL seen in normal, mast cell-positive control mice was found to be absent (Kamiya, Oku, Fukomotu and Ooi, 1983). Links between the cell types in sheep were also confirmed by histochemical and ultrastructural studies carried out on MMC, TC and GL isolated from the abomasa of animals challenged with *T. circumcincta* (Huntley, Newlands and Miller,1984a).

The presence of elevated numbers of GL in sheep is normally associated with repeated nematode infections and resistance to challenge (O'Sullivan and Donald 1973, Stankiewicz, Pernthaner, Cabaj, Jonas, Douch, Bisset, Rabel, Pfeffer and Green, 1995). In the goat, the association between resistance to challenge and the presence of relatively large numbers of GL appears to be weaker, since they harbour large parasite burdens despite an abundant population of mucosal GL (Huntley *et al.*, 1995). This lack of correlation suggests that caprine GL may be less effective than ovine GL in any effector function against nematodes, or that they are not important in the process. During the present studies efforts will be made to determine whether goat GL are also derived from degranulated MMC, since their continued presence in the face of the large numbers of retained worms (Le Jambre and Royal, 1976; LeJambre, 1984, Huntley *et al.*, 1995) may indicate deficiencies in MMC/GL-associated immune mechanisms against nematodes in this species.

1.3.6 Comparative mucosal mast cell/globule leukocyte responses in goat and sheep nematode infections

To date, only one study has been carried out to measure differences in the functional activity and numbers of MMC/GL in goats, sheep and lambs infected with

T. circumcincta and *T. vitrinus*. (Huntley *et al* 1995). In this experiment the putative inter-species cross reactivity of a polyclonal antibody to SMCP was utilised to compare the responses, both immunohistochemically in tissue sections and by immunoassay of SMCP concentrations in abomasal and jejunal homogenates. SMCP analyses were accompanied by conventional histochemical assessments of MMC and GL numbers achieved with toluidene blue (Enerback, 1966a) and carbol chromotrope (Lendrum, 1944) stains respectively. Cellular responses were considered in relation to the worm burden carried by the goats and sheep in order to determine whether levels of MMC/GL activity might have any bearing on parasite survival in the host.

The results confirmed previous findings (LeJambre and Royal, 1976; LeJambre, 1984) that adult goats are more susceptible to nematode infections than adult sheep, with significantly higher total worm burdens being recovered from the goat abomasal and jejunal tissues after challenge. Results for mast cell numbers and protease levels in the abomasum and jejunum also showed fundamental differences between the two species. In goats there were larger numbers of GL with relatively few MMC compared to the sheep, whilst substantially higher SMCP concentrations were demonstrated in the gastro-intestinal tissues of sheep compared to the goats. This may indicate that the presence of increased mast cell protease concentrations is a significant component of the acquired immune response to nematodes and, although goats showed a significant increase in GL, their relative lack of MMC and significantly lower concentrations of putative granule SMCP-like protease may have contributed to their continued susceptibility.

1.4 Aims

Work arising from this thesis will hopefully resolve some of the questions regarding the functional capabilities of goat MMC and GL through the development of specific, homologous techniques for the detection and characterisation of a putative goat mast cell protease (GMCP). This will allow *in vivo* and *in vitro* assessments of mast cell responses in goats undergoing infections with GI nematodes and will be achieved as follows:

1) The purification, biochemical characterisation and raising of antibodies to a goat mast cell protease (GMCP) isolated from nematode-infected goat intestinal tissues.

2) The immunological and histological characterisation of GMCP; determining its immunoreactivity with SMCP as well as defining its distribution within goat mast cell and GL populations from a variety of tissues.

3) The molecular characterisation of GMCP by the sequencing of cDNA isolated from goat BMMC.

4) Experiments examining the *in vivo* functional activity of mast cells in goats and sheep undergoing both primary and secondary infections with *T. circumcincta*.

5) *In vitro* generation of caprine BMMC, with the recombinant ovine cytokines and *ex vivo* isolation of MMC from parasitised goat intestinal tissues enabling their morphological, ultrastructural and functional characterisation.

6) Functional activation studies on goat and sheep BMMC to examine whether granule-associated mediator release occurs after their exposure to a variety of nematode antigen preparations *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Buffer recipes

All chemicals used (unless indicated otherwise) were analytical or general purpose reagent grade purchased from BDH Ltd. or Sigma, Poole, Dorset, UK. Recipes for the buffers, culture media, fixatives and histochemical stains are given in detail in Appendix A 'Buffers and Fixatives'.

2.2 Procedures involving the use of animals

Animal use was kept to a minimum throughout this study. Where they were necessary, all procedures were carried out in strict accordance with Home Office guidelines as covered by Project Licence No. PPL 60/01464 and Personal Licence No. PIL 60/5750.

2.3 GMCP purification

2.3.1 Sample handling

To minimise catalytic breakdown of GMCP, all tissues, purification fractions and enzyme preparations were maintained on ice at all times throughout the purification procedure. FPLC purification steps were also completed, wherever possible, in a single day to reduce sample degradation by repeated freeze-thawing.

2.3.2 Animals

Gastro-intestinal tissue from Angora cross 'Scottish cashmere' goats aged six months to four years were used for all GMCP purifications. The majority were from Sourhope farm, Kelso, Roxburghshire, Scotland. Following natural exposure to nematodes at pasture, they had been housed and challenged according to an anthelmintic disrupted challenge model (ADCM) commonly used in challenge studies conducted at Moredun Research Institute (MRI) (Smith *et al.*, 1983 and 1985; Coop, Huntley and Smith, 1995; Huntley *et al.*, 1995; Patterson, 1996). This regime involved

treating the animals with anthelmintic (fenbendazole (Panacur) 10 mg/kg) or ivermectin (Ivermectin (Ivomec injectable) 400µg /kg) and housing them before administering a 'trickle challenge' infection consisting of 2000 *T. circumcincta* L_3 and 1000 *T. vitrinus* L_3 five times weekly for 4 to 8 weeks. The goats were then treated with anthelmintic again before being given a secondary challenge with 50,000 T. circumcincta L_3 seven days later. Ten days after this second challenge, they were killed and the tissues harvested.

2.3.3 Tissue collection and preparation

Tissue collection

Animals were killed either by captive bolt stunning and exsanguination or by intravenous injection of 20mls pentobarbitone solution (Euthatal, Rhone Mérieux Ltd.). Immediately post mortem, the abomasum and a section of jejunum (approximately 50 cm long and taken distal to a point starting 1 metre below the pylorus), were removed, everted and washed briefly in tap water to remove any residual intestinal contents. (In one experiment, tissues from other sites were also removed, see Chapter 4). The tissues were placed in labelled plastic bags and transported, on ice, to the MRI where they were stored at -70°C prior to processing.

ELISA homogenate preparation

Tissues for ELISA analysis (see 2.12 below) were trimmed to portions weighing approximately 1g and transferred to pre-weighed sterile universals. The universals were also transported to MRI on ice where they were weighed again to ascertain the actual tissue weight before adding ten volumes of 20mM Tris HCl + 1.5M NaCl pH 7.5. The samples were homogenised in a Polytron homegeniser (Cave, North Humberside, England) and the homogenate stored at -20 °C until analysis.

Tissue homogenate preparation for protease purification

5-7 g aliquots of tissue for GMCP purification were thawed and homogenised in 3 volumes of 20mM tris HCl pH 7.5 and centrifuged at 600 x g for 20 minutes in a Beckman J2-21 centrifuge. The supernatant was removed and the pellet resuspended in 2 volumes of 1.0M NaCl 20mM Tris HCl+0.1% Brij 35 pH 7.5 (Brij 35, Sigma Cat. no. 430 AG-6, was added to all subsequent buffers to prevent adherence of GMCP to vessel surfaces) before centrifuging at 4 ^{o}C for 1 hour at 20,000 x g. The resulting supernatant, termed the 1.0M NaCl tissue extract, was collected and further clarified by centrifuging at 4 ^{o}C for another hour at 20,000 x g.

2.3.4 Active protease monitoring in samples

Tissue homogenates and samples taken throughout the purification process were tested for their proteolytic activity using synthetic, chromogenic substrates. Carboxybenzoyl-L-tyrosine nitrophenol ester (Cbz-L-Tyr-NPE) (Sigma Cat. No. C 3637) was used in the initial stages of the project, but subsequently, carboxybenzoyl-L-lysine thiobenzyl ester (BLT) (Sigma Cat. No. C 3647) was used due to its more rapid rate of hydrolysis in the presence of GMCP. Samples (2µl) to be tested were added to 46 µl of 0.1M Tris HCl pH 7.8 containing 2µl of 5mM Cbz-L-Tyr-NPE dissolved in dimethyl sulfoxide (DMSO) (Sigma Cat. No. D 5879) or 44µl of 0.1M Tris HCl pH 7.8 containing 5mM BLT stock solution + 5mM dithionitrobenzoic acid (DTNB) (Appendix A) in DMSO. Positive samples produced a yellow colour within five minutes at room temperature.

2.3.5 Fast Protein Liquid Chromatography (FPLC) separation of tissue homogenate samples

Sample purification was achieved by sequential affinity and cation exchange chromatography using columns attached to a Pharmacia Bio-tech FPLC system. This consisted of two P-500 pumps (Pharmacia Bio-tech, Uppsala Cat. No. 18-1003-65)

termed A and B which controlled the relative amounts of initial sample buffer (buffer A) and elution buffer (buffer B) passing through the column. Fall through and eluted samples from the columns were collected into an automated rotary fraction collector (Pharmacia Bio-tech, Uppsala Cat. No. 18-1000-77) maintained at 4 ^{o}C by the circulation of refrigerated H_2O. The total protein concentrations of these samples were measured at 280nm using a UV-1 monitor (Pharmacia Bio-tech, Uppsala Cat. No. 18-1003-66) connected to a REC 101 paper chart recorder (Pharmacia Bio-tech, Uppsala Cat. No. 18-1001-42) . Homogenate (10mls) was initially diluted 1:3 in 20 mM tris HCl + 0.1% Brij 35 pH 7.5 and the total volume applied to a column packed, according to manufacturer's instructions, with 25mls CM-sepharose CL-6B gel (Pharmacia Bio-tech, Uppsala Cat. No. 17-0720-01). Bound sample was eluted with a 0-1.0M NaCl gradient in 20mM trisHCl 0.1% Brij and the resulting fractions tested for substrate activity. Positive fractions were pooled and diluted 1:3 in 20mM trisHCl 0.1% Brij 35 pH 7.5 prior to loading onto a pre-packed 2ml monoS HR 5/5 cation exchange column (Pharmacia Bio-tech, Uppsala Cat. No. 17-0547-01). Bound sample was eluted with a stepped 0-0.5 M NaCL 20mM trisHCl pH 7.5 0.1% Brij gradient. Peak fractions which caused the most rapid substrate hydrolysis were collected and pooled, prior to concentrating to a volume of less than 1ml in Centricon-10 (Amicon Ltd., Stonehouse, UK) small volume centrifugal concentrator tubes. Samples were taken from each purification step and stored at -70 ^{o}c prior to testing by SMCP ELISA for protease content and a commercial bichinoic acid (BCA) assay (Pierce corporation, USA Cat No. 23225) for total protein content.

2.3.6 Silver stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

This was carried out using a modification of Laemmli's technique (Laemmli 1970). Resolving gels (12% or 7.5%) were made up as outlined in Appendix A and immediately cast into BioRad Mini-Protean II SDS-PAGE gel apparatus (BioRad systems Cat. No. 165-2940). 1ml of 70% ethanol in double distilled (dd) H_2O was layered on top of the gel mixture and the gels left to set for 20 minutes at room temperature. Meanwhile, a 4% stacking gel mixture was also made up (Appendix A) . Once the resolving gel portion had set, the 70% ethanol was poured off and the stacking gel layered onto the resolving gel. Sample well combs with space for 10 or 15 samples per gel were inserted into the stacking gel, which was left to set for a further 10 minutes at room temperature.

Samples for SDS-PAGE were diluted in either reducing or non-reducing (see 2.3.7) sample buffer (Appendix A) prior to loading. Samples dissolved in reducing sample buffer were heated to 100 ^{o}C in a water bath for 3 minutes to denature the sample proteins. The presence of glycerol and bromophenol blue in the sample buffers facilitated sample loading onto the gels and also allowed the position of the sample to be monitored throughout the procedure. Following immersion of the gel tank in 500 mls of tank running buffer (Appendix A), 5-7 µl of BioRad reference protein standards (BioRad systems Cat. No. 161-0304) consisting of *Escherichia coli* galactosidase (116,000 kD); phosphorylase b (97.4 kD); bovine serum albumin (66.2 kD); chicken egg ovalbumin (42.7 kD); carbonic anhydrase (31.0 kD) and soyabean trypsin inhibitor (21.5 kD) were used as molecular weight standards.These were diluted in the same sample buffer as the unknown samples and loaded into the furthest left sample well, followed by 10-20 µl aliquots of the unknown samples in the subsequent wells. Gels were run at 200 volts for 45-50 minutes, or until the bromophenol blue dye front had reached the bottom of the gel.

Protein bands in samples, thus separated by SDS-PAGE were visualised by dismantling the apparatus and staining the gels with silver nitrate using the following protocol modified from Morrissey (1981):

1. 30 minutes fixation in 50% methanol and 10% acetic acid
2. 30 minutes fixation in 5% methanol and 7% acetic acid
3. Three 5 minute washes in ddH_2O to rehydrate the gel
4. 20 minutes wash in 5µg/ml dithiothreitol in ddH_2O to completely reduce the electrophoresed proteins
5. 30 minutes wash in 0.1% silver nitrate
6. Rinse briefly in ddH_2O
7. Develop in 3% sodium carbonate (Na_2CO_3) containing 50µl 37% paraformaldehyde per 100mls solution until adequate staining is obtained.
8. Stop the development by adding 5mls 2.3M citric acid and agitating the gel for 5 minutes.

2.3.7 Azocasein substrate gels

Non-reduced protein samples were loaded onto 12% resolving SDS-PAGE gels incorporating 20mg of azocasein substrate (Sigma Cat. No. A 2765) (AAppendixA). The gels were prepared and run out as described above. The gels were given two 45 minute washes in PBS + 2.5% Triton X-100 (Sigma Cat. No. x 100) to remove the SDS from the gel matrix. They were then given three 5 minute washes in PBS before being incubated overnight at 37 ^{o}C in phosphate buffered saline (PBS) + 0.01% sodium azide (NaN_3). Staining for 30 minutes in 0.5% Coomassie Blue (Appendix A) was followed by three 10 minute washes in destainer solution (Appendix A) to remove the excess Coomassie Blue. Areas of proteolytic activity could be seen as clear bands against a blue background, due to enzymatic cleavage of the casein component of the substrate.

2.4 Production of antibodies to GMCP

2.4.1 Rabbit polyclonal antibody production

Immunisation

Polyclonal antibodies were raised by inoculating New Zealand White (NZW) rabbits, with aliquots of purified GMCP emulsified 1:1 with Freund's adjuvant. Each animal received a total volume of 0.5mls per injection, which was administered subcutaneously as a divided dose on the left and right flanks. The immunisation protocol consisted of a primary injection of approximately 10μg GMCP in Freund's complete adjuvant (Sigma Cat. No. F-5881), followed by at least two booster injections of approximately 10μg GMCP in Freund's incomplete adjuvant (Sigma Cat. No. F-5506) at two to three week intervals thereafter. Test bleed sera were taken from the caudal marginal ear vein 10 days after the final booster and tested for activity against purified GMCP dot blotted onto nitrocellulose.

Harvesting of sera

Sera positive for activity against GMCP was collected by exsanguination by cardiac puncture under terminal intra-venous anaesthesia (Sagatal, Rhone Mérieux Ltd.). Harvested blood was allowed to clot in sterile universal tubes for 4 hours at room temperature before spinning at 600 x g for 10 minutes. The resulting serum supernatant was collected and divided into 1ml or 5ml aliquots for storage at -70 ^{o}C.

Protein A purification of serum IgG

The IgG-containing fraction of the rabbit serum was purified by column chromatography using a 5ml Protein A sepharose CL-4B column (Pharmacia Bio-tech, Uppsala Cat. No. 17-0780-01) attached to the FPLC apparatus described above. Aliquots of serum (2mls) were diluted 1:5 in 20mM Tris/HCl + 0.5M NaCl pH 7.5 and

loaded onto the column, the fall through fraction being collected for subsequent testing. The bound IgG fraction was eluted with 10mls of 0.1M citrate pH 2.2 and immediately neutralised to pH 7.5 by adding 1M Tris buffer to the fraction containing the eluted protein peak. Both the fall through and eluted samples were tested by dot blotting against purified GMCP, and immunohistochemistry on goat jejunal tissue sections (see below), to ensure that the eluted fraction contained all the antibody specific for GMCP. The total protein content of the eluted sample was determined by BCA assay (Pierce corporation, USA Cat. No. 23225) and adjusted to 1mg/ml before being divided into 50µl aliquots for storage at -20 ^{o}C.

2.4.2 Mouse monoclonal antibody production

Immunisation

Male and female Balb/c mice were immunised subcutaneously on three occasions, three weeks apart, with 100 µl sterile PBS containing approximately 5µg purified GMCP and 20µg QuilA (Superfos Biosector Batch No. C77-67) adjuvant per injection. Tail bleeds were taken ten days after the last injection and tested for activity against purified GMCP by dot blotting. Animals containing anti-sera positive for GMCP were immediately given a final intra-peritoneal booster injection of the same mixture. Three days later, they were killed by cervical dislocation and their spleens removed under aseptic conditions for splenic lymphocyte harvesting.

Splenic lymphocyte harvesting

Harvested mouse spleens were placed in sterile petri dishes in RPMI 1640 medium + 10% FCS + P/S (Appendix A) (Kennet, Denis, Tung and Klinman, 1978). Several holes were made in the splenic capsular tissue with a 26g needle before the spleen was gently injected with 5mls medium to displace the parenchymal cells into the surrounding dish. The resulting cell suspension was centrifuged at 200 x g for five

minutes and the supernatant removed. The cell pellet was gently resuspended, washed twice in serum free RPMI 1640 + P/S and adjusted to 1-2 x10^7 cells/ml.

Hybridoma fusion protocol

The method was a modification of that used by Claflin and Williams (1978). 1 x 10^7 or more NS0/1 murine myeloma cells (ECACC No. 85110503) were grown in 225 cm^3 canted neck tissue culture flasks (Corning Cat. No. 25110-75) in serumless RPMI 1640 medium + P/S (Appendix A) from liquid nitrogen stored stocks for three to five days prior to carrying out the fusion protocol (Galfre and Milstein, 1981). These mutant myeloma cells are defective for hypoxanthine guanine phosphoribosyl transferase (HGPRT) which prevents their growth in selection medium containing the antifolate drug aminopterin. After fusion with murine spleen cells containing HGPRT, the salvage pathway for the synthesis of purine and thymidylate is enabled in the hybridoma cells. This results in the growth of immortalized hybrids which can successfully synthesise nucleotides derived from purine and thymidylate. In contrast, residual unfused Y3 and non-immortalized spleen cells are either killed by the continued presence of aminopterin or die naturally during the subsequent two weeks culture in serum-free medium. The Y3 and splenic lymphocyte cells were mixed to give a 1:5 cell ratio and centrifuged at 200 x g for 5 minutes. The supernatant was removed and the cell pellet tapped loose before resuspension in 0.5ml 35% Hybri-max® poly-ethylene glycol (PEG) (Sigma Cat. No. P 7181). The suspension was centrifuged at 100 x g for three minutes. Eight minutes after the addition of the PEG, 5 mls serum free RPMI 1640 + P/S was added slowly over two minutes without disturbing the pellet. The cells were then gently resuspended by swirling over a further three to four minutes, taking care not to dislodge the whole pellet at once. The suspension was centrifuged at 200 x g for five minutes, the supernatant removed and 5mls RPMI 1640 + oxaloacetic acid (OAA) + pyruvate (PYR) + insulin (INS) +

hypoxanthine, aminopterin, thymidine (HAT) + 3-[N-morpholino]propanesulphonic acid (MOPs) + 10% foetal calf serum (FCS) (plating medium, Appendix A) was added slowly without disturbing the pellet. Seven minutes later, the pellet was gently resuspended and the resulting suspension made up to 60 mls with more plating medium. The final suspension containing the hybridoma cells was plated out in 100µl aliquots onto six sterile 96 well plates (Corning, USA Cat. No. 3595) before incubating at 37 ^{o}C in a tissue culture incubator (ICN Flow automatic CO_2 Incubator Model 160, ICN Pharmaceuticals) in a humidified atmosphere of 5% CO_2 in air.

Production of stable antibody producing clones

The hybridoma cells were fed every two to three days by removing the medium along with any dead and unfused cells and replacing with 100µl fresh RPMI 1640 + OAA + PYR + INS + HAT + MOPS + 10% FCS. After seven days, the plates were screened for the presence of hybrid colonies, which could be identified under a Leica DM 1L inverted microscope (Leica microscopy Cat. No. 520802/152192) as round colonies of bright, rapidly dividing cells. The location and number of colonies in each well was recorded and their growth monitored daily until the cells covered approximately 1/3 of the well surface area. Supernatants from these wells were assayed for the presence of antibody to GMCP by tissue immunohistochemistry (see 2.8.7 below), and positive antibody-secreting hybrids were resuspended by aspiration, for sub-cloning into fresh 96 well plates. The GMCP antibody-positive hybrid cells were sub-cloned by limiting double dilutions of the cell suspensions in RPMI 1640 + OAA + PYR + INS + MOPS + 10% FCS (without the addition of HAT), to ensure that individual cell colonies in the sub-cloned plates were derived from single parent clones (Goding 1983). Supernatants from all wells containing single colonies were again tested, by immunohistochemistry, for the presence of antibody to GMCP and, following the second sub-cloning, positive hybrids plated out into sterile 12 well plates

(Costar Cat. No. 3523) and fed with RPMI 1640 + OAA + PYR + INS + MOPS + 10% FCS. These cells were grown to confluency and split into further wells to produce sufficient cell stocks for storage in liquid nitrogen as well as injection into Balb/c mice for production of ascitic fluid.

Liquid nitrogen storage of antibody producing clones

Cells for storage in liquid nitrogen were harvested from the 12 well culture plates by aspiration and centrifuged at 200 x g. The supernatant was removed and the cells resuspended in 1-2 mls 'freezing down' medium containing 50% FCS, 40% RPMI 1640 + P/S and 10% dimethyl sulphoxide (Hybri-max® DMSO, Sigma Cat. No. D 2650) before storing in 1ml freezing vials (Sigma Cat. No. V 4381) for slow freezing in a nalgene container ('Mr. Frosty' Sigma Cat. No. C 1562) overnight at -70 ^{o}C. Once frozen, the tubes were transferred to dewars containing liquid nitrogen for long term storage. Tubes containing cell aliquots removed from liquid nitrogen storage were rapidly thawed in a water bath at 37 ^{o}C and their contents diluted 1: 20 in RPMI 1640 + OAA + PYR + INS + MOPS + 10% FCS. The cells were washed twice in the same medium before setting up at 2 x 10^5/ml in flasks or plates.

Ascites production

Five days after priming with a subcutaneous injection of 100µl pristane (0.79g/ml 2,6,10,14-tetramethyl-pentadecane, Sigma, Cat. No. T-7640), Balb/c mice were injected intra-peritoneally with 5 x 10^5 cells in 0.5 mls PBS and monitored daily for the development of ascites. This usually occured approximately ten to twenty one days after injection. Ascitic animals were killed by cervical dislocation and the ascitic fluid harvested (generally 1-5mls per animal) under aseptic conditions using a 16g needle attached to a 5ml syringe. The ascitic fluid was centrifuged at 600 x g for ten minutes and the supernatant stored in 1 ml aliquots at -20 ^{o}C

Protein G purification

Ascites fluid Ig purification was carried out as for the polyclonal rabbit anti-sera to GMCP using Protein G-sepharose (Pharmacia Biotech Cat No. 17-0618-02) in place of protein A-sepharose. Both fall through and eluted fractions were tested for positive staining of tissue sections to ensure that antibody binding to the column had occurred. A BCA assay (Pierce corporation, USA Cat. No. 23225) was carried out to quantify the protein content of the eluted antibody which was adjusted to 100 µg/ml in 20mM Tris/HCl pH 7.5.

Biotinylation protocol

Protein A eluted monoclonal antibody was dialysed against 0.1M $NaHCO_3$, pH 8.4, overnight at 4 ^oC and the protein concentration adjusted to 1mg/ml. N-hydroxysuccinimidobiotin (Sigma Cat. No. H 1759) was dissolved in DMSO (Sigma Cat. No. D 5879) at 1mg/ml and 120µl of the biotin ester was added to each ml of monoclonal antibody solution (Goding, 1983). This mixture was incubated at room temperature for 4 hours then dialysed against PBS + 0.01% NaN_3 overnight at 4 ^oC. The biotinylated antibody solution was diluted 1:1 in glycerol as a cryoprotectant and stored at -20 ^oC.

2.5 Dot blotting and Western blotting on nitrocellulose

2.5.1 Dot blotting

Samples (2µl) containing GMCP, SMCP or IgE (see 2.13, below) were blotted, by pipetting, onto a nitrocellulose (NC) membrane (Bio-blot, Costar, Cat No. 8801) and allowed to dry for fifteen minutes at room temperature. The NC membrane was blocked for thirty minutes in two changes of PBS + 0.5M NaCl + 0.5% Tween80 (Appendix A), which was also used as a reagent diluent and wash buffer for all

subsequent steps. The blot was incubated with primary antibody diluted 1:200 or 1:500 for 1 hour at room temperature and given three five minute washes, before incubating for a further hour with the appropriate peroxidase-linked conjugate antibody or biotinylated secondary antibody diluted 1:500. Blots probed with a biotinylated secondary antibody were washed as above and treated for a further thirty minutes with peroxidase-linked streptavidin (Streptavidin-POD; Boerhinger Mannheim Cat. No. 1 089 153) diluted 1:2000. Antibody binding to the protein sample was visualised by washing the blots and staining for five minutes with 3, 3'-diaminobenzidene in H_2O_2 (Sigma Fast™ DAB tablets; Sigma Cat. No. D 4293). Controls consisted of blotted samples incubated with either normal serum or a non-cross reactive monoclonal antibody diluted 1:200 or 1:500 in place of primary polyclonal or monoclonal antibodies respectively.

2.5.2 Western blotting

This was performed using a three-buffer modification of the Khyse-Anderson semi-dry technique for protein transfer onto nitrocellulose membranes (Towbin & Gordon, 1984). SDS-PAGE was carried out on 12% gels loaded with reduced samples as described previously. Gel sized double layers of filter paper (3M paper; Whatmans Cat No. 3030917) were soaked in anode buffer 1, anode buffer 2 and cathode buffer (Appendix A). Gel sized sections of NC membrane (Bio-blot NC, Costar, Cat. No. 8801) were cut out and soaked in anode buffer 2. The SDS-PAGE gels were placed on top of the NC membranes, anode buffer 2 and cathode buffer soaked 3M papers in a blotting apparatus (Sigma Cat. No. B 2529), as shown in Fig. 2.1. The positions of the gels and sample lanes were marked on the NC membrane before covering with the anode buffer soaked 3M papers. The apparatus was closed with the anode plate and attached to a constant current power supply. A transfer current of 70mA per gel was applied to the apparatus for ninety minutes before

dismantling and removing the blotted NC membrane. The molecular weight standard lanes were cut from the remainder of the blot and stained for one minute in a 0.1% solution of amido black (Sigma Cat. No. A 8181) before washing in double distilled (dd) H_2O for thirty minutes to remove excess stain. Sample-containing lanes for immunostaining were cut out as necessary and treated with the appropriate antibodies as outlined previously for dot blots.

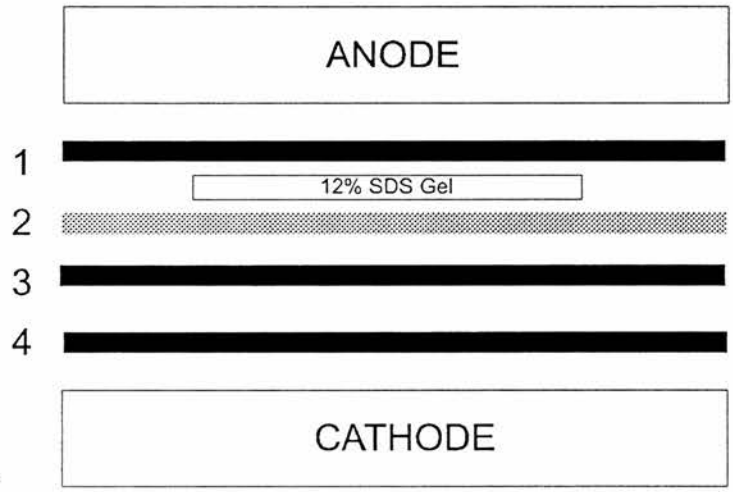


Fig. 2.1 Diagram of assembled Western blot apparatus.
1) 3M papers soaked in anode buffer 1.
2) Nitrocellulose membrane soaked in anode buffer 2.
3) 3M papers soaked in anode buffer 2.
4) 3M papers soaked in cathode buffer. Sample proteins were transferred to the NC membrane using a current of 70mA per gel for ninety minutes

2.6 Characterisation of GMCP

2.6.1 Synthetic substrate assays and inhibitor assays

Synthetic substrate assays were based on the method described in 2.3.4 for monitoring GMCP purification. For substrate assays, GMCP was compared with SMCP, bovine α-chymotrypsin (Sigma Cat. No. C-7762) and bovine pancreatic trypsin (Sigma Cat. No. T-8918). Stock enzyme solutions were diluted in 0.1M Tris

HCl + 0.1% Brij 35 pH 7.5 to provide the same concentrations for each preparation (1 - 1.5 µg/ml). Substrate stocks were obtained from Sigma and made up to 5mM solutions in DMSO. (See Appendix A for details of the substrates used). Each substrate was freshly prepared prior to each test. For BLT, where DTNB was the chromogen, the components were made up separately and mixed just prior to adding to the reaction mixture. To 40µl of 0.1M Tris HCl + 0.1% Brij 35 pH 7.5 in a microtitre plate well (Dynatech, Cat No. M129A) was added 5µl of enzyme followed by 5µl of substrate solution. For the thiobenzyl ester and nitrophenol ester substrates, the reaction was allowed to proceed for five minutes at room temperature, whilst the nitro-anilide substrates were incubated for two hours in a warm air oven at 37 ^{o}C (A. Pemberton, personal communication). All reactions were terminated by adding 100µl 100% methanol. The degree of colour change was measured at 492nm on a Titertek MC scanner and the readings corrected for colour changes in parallel negative control wells containing buffer and substrate only.

Enzyme kinetic studies to evaluate the Michaelis-Menten constant (Km) for GMCP and SMCP in the presence of BLT were also carried out using a Beckman DU-650 spectrophotometer. The enzyme assays were set up as previously described for the microtitre plate assays in 100 µl capacity quartz glass cells (Beckman microcells). Each reaction was carried out with reducing concentrations of substrate added to each reaction mixture in the presence of 1µg/ml GMCP. Freshly prepared aliquots of substrate were double diluted in DMSO (Sigma Cat. No. D 5879) to provide a stock concentration range of 1.56-50 mM. Aliquots (2µl) of these stock solutions were added to the enzyme assay mixture to provide a final reaction concentration range of 0.125-2mM . The reactions were monitored for between sixty seconds and three minutes at 405nm, and the rate of substrate hydrolysis at each substrate concentration averaged over a minimum of three reactions. The Km values

for the substrates were calculated from the initial rates of substrate hydrolysis, using onboard enzyme mechanism analysis software supplied with the spectrophotometer.

For inhibitor assays, samples of GMCP and SMCP (1.5 µg/ml) were incubated for one hour at room temperature with the following small molecular weight inhibitors (final reaction concentration shown in brackets); 1,10 phenanthrolline (50mM, Sigma Cat. No. P-9375), pepstatin (5mM, Sigma Cat. No. P-4265), l-*trans*-epoxysuccinylleucylamido-(4-guanido)-butane (E64) (0.05mM, Sigma Cat No. E-3132), phenylmethylsulfonylfluoride (Pms-F) (5mM, Sigma Cat No. P-7626). In addition, samples were incubated with goat serum and sheep serum respectively (1: 200 dilution in 0.1M tris HCl). The samples were assayed for residual proteolytic activity against BLT as described above. The results were expressed as percentage inhibition compared to positive control samples of enzyme pre-incubated in 20 mM Tris buffer pH 7.5 only.

2.6.2 Amino acid sequencing of immunoaffinity purified GMCP

Protein A purified rabbit IgG antibodies to GMCP (5mg) were coupled to 1ml cyanogen bromide activated sepharose 4B (CNBr-sepharose) (Pharmacia Biotech, Uppsala Cat. No 17-0430-01) according to the manufacturer's instructions. This was used to construct an immunoaffinity column for the extraction of GMCP from a 20mM Tris HCl + 1.5M NaCl extracted jejunal homogenate sample. Samples (5g) of tissue were extracted in three volumes of 20mM TrisHCl +1.5M NaCl+0.1% Brij 35 pH 7.5 and centrifuged as described in 2.3.3. The extract was then incubated for forty five minutes at room temperature with 2mM Pefabloc^{tm} (Boerhinger Mannheim Cat. No. 1 429 868) broad spectrum protease inhibitor to minimise proteolytic breakdown of the column bound IgG. Small (1-3ml) aliquots of the resultant sample were diluted 1:3 in 20 mM Tris/HCl+0.1% Brij 35 pH 7.5 and applied to the column sequentially. Bound sample was eluted as for the protein A column described previously (2.4.1).The

eluted protein samples were pooled and concentrated under vacuum in a dialysis thimble (Sartorius Cat. No. 250-11) immersed in 20 mM Tris/HCl+0.1% Brij 35 pH 7.5 at 4 ^{o}C, then reduced and run on 12% SDS-PAGE gels. The gels were blotted onto an Immobilon-P membrane (Millipore Cat. No.P 0807) pre-soaked in 100% methanol using the three buffer method outlined above. Blotted proteins were stained with 0.5% Coomassie Blue (Appendix A) for five minutes before destaining with two or three rapid washes in 100% methanol to reveal blue coloured protein bands. The blots were allowed to dry at room temperature before cutting out bands with a molecular weight corresponding to GMCP. These were sent to the Microchemical Facility, Babraham Research Institute, Cambridge, England for NH_2-terminal amino acid sequence analysis.

2.7 Cloning of cDNA sequences for GMCP

2.7.1 Total RNA extraction from goat BMMC

Aliquots (5 x 10^6 cells) of goat bone marrow derived mast cells (BMMC) cells grown in recombinant ovine interleukin-3 and recombinant ovine stem cell factor (see below), were collected on days 2, 5 and 7 of the cultures. They were spun at 200 x g for five minutes lysed in 1ml Tri-reagent (Sigma Cat. No. T 9424) and then stored at -70 ^{o}C. The total RNA was obtained by sequentially extracting the cell lysate in chloroform and isopropanol. The isopropanol fraction containing precipitated RNA was centrifuged at 13000 rpm for five minutes and the pellet washed in 75% ethanol. The sample was re-centrifuged and the RNA pellet allowed to air dry before dissolving in 50µl DNase/RNase free, sterile ddH_2O (Sigma Cat. No. W 1754).

2.7.2 Messenger RNA (mRNA) selection from total RNA

This was carried out using a commercial kit (Quickprep Micro mRNA Purification Kit; Pharmacia Bio-tech, Uppsala Cat. No. 27-9255-01) consisting of microspin® columns packed with oligo(dT)-cellulose. The solubilised total RNA preparations were passed through the columns which selectively bound the 3' poly-adenosine (poly-dA) tail specific to mRNA, allowing it to be eluted free from contamination with ribosomal RNA and genomic DNA.

2.7.3 Formaldehyde gels for RNA detection

1% agarose gels containing formaldehyde, for the detection of total and mRNA extracted from BMMC, were made up as described in Appendix A. RNA samples or RNA markers (0.1mg/ml) (Boerhinger Mannheim Cat. No. 1 062 611) (6 µl) were added to 12.5µl formamide (Sigma Cat. No.F 7508), 2.5µl 10 x MOPS (Sigma Cat. No.M 5162) and 4µl 37% formaldehyde. The mixture was incubated for ten minutes at 65 ^{o}C then 2.5µl Northern running dye (Appendix A) and 0.5µl of 0.5mg/ml ethidium bromide (Sigma Cat. No. E 1385) added. The gels were loaded with 20µl of sample per well. Separated bands were visualised on a dual intensity ultra-violet (UV) transilluminator (Hoefer Scientific Instruments).

2.7.4 Reverse transcriptase reaction protocol

cDNA templates for sequencing the central and 3' fragments of GMCP were constructed by priming the purified RNA with either a random hexamer primer or a specially synthesised poly-thymidine primer kindly provided by Dr. D. Knox. This latter primer is specific for mRNA 3' poly-dA nucleotide sequences. In contrast, cDNA templates for the 5' GMCP fragment were primed with an ovine P7 primer (see chapter 5, Fig. 5.1) to ensure maximal quantities of 5' specific cDNA in the final reaction mixture. Both templates were then constructed by incubating the RNA and

the template primers with 20 units of cloned M-MuLV (murine leukaemia virus) reverse transcriptase enzyme (Boerhinger Mannheim Cat. No. 1 062 603) in the presence of a 2mM solution of mixed nucleotides (dNTPs) at 42 ^{o}C for sixty minutes.

2.7.4 Primary and secondary polymerase chain reaction (PCR) amplification using nested primers for GMCP and SMCP

Primer oligonucleotides specific for GMCP and SMCP cDNA sequences were commercially synthesised (Cruachem, Glasgow). Amplification of GMCP cDNA required the use of nested or semi-nested primers (Sambrook, Fritsch & Maniatis 1989), for 30 cycle primary and secondary polymerase chain reactions (PCR) which were carried out on an Astec pc-700 thermocycler in the presence of 1.0 unit of *Thermus aquaticus* BM, recombinant, *E.coli* Taq DNA polymerase (Boerhinger Mannheim Cat. No. 1 647 679) (see Appendix A for PCR reagent volumes and the amplification programme). The Taq polymerase was added at 72 ^{o}C as a 'hot start' following an initial 10 minute cDNA strand denaturing step at 94 ^{o}C.

2.7.6 Agarose gels for PCR product detection

Agarose gels (0.8%) for the detection of PCR amplified cDNA were made up as described in Appendix A. 10 µl of PCR product or 1 kilobase pair (kb) DNA standard ladder (0.1mg/ml) (Boerhinger Mannheim Cat. No. 236 233) was combined with 2 µl agarose gel dye (Appendix A) and the total volume loaded onto the gel. Following electrophoresis, separated bands were viewed on a dual intensity UV transilluminator (Hoefer Scientific).

2.7.7 Southern blotting protocol

Agarose gels containing PCR product cDNA were denatured, neutralised (AppendixA) and southern blotted overnight onto Hybond N+ nylon membranes (Amersham International, Cat. No. RPN 303B) according to the manufacturer's

instructions. This allowed subsequent probing with digoxigenin-labelled oligonucleotide primer sequences specific for the required amplified cDNA fragments.

2.7.8 Digoxigenin-labelling (dig-labelling) of oligonucleotide probes

Mast cell protease-specific oligonucleotide sequence probes were labelled with a digoxigenin ddUTP molecule according to the manufacturer's instructions using a DIG-oligonucleotide 3'-End labelling Kit (Boerhinger Mannheim Cat. No. 1 362 372). 20µl of labelled probe was diluted in 10mls Rapid-hyb™ (Amersham International Cat. No. RPN 1636) and stored at -20 ^{o}C between uses.

2.7.9 Probe hybridization protocol (see Appendix A for buffer details)

Blotted Hybond N+ membranes were rinsed in 6 x saline sodium citrate (SSC), dried for thirty minutes and fixed by cross-linking with two 1200J pulses of ultra violet light (Hoefer UV cross-linker, Hoefer scientific). Lanes containing the DNA molecular weight markers were removed and stained with Genogold® (British Biocell Cat. No. Gen 500) according to the manufacturer's instructions. The remaining portions of the blots were incubated for forty five minutes at 40 ^{o}C in a hybridisation oven (Techne Hybridiser HB-1D) in 10mls Pre-hybridisation buffer (Rapid-hyb™ Amersham International Cat. No.RPN 1636), followed by 3 hours incubation at 40 ^{o}C with the dig-labelled oligonucleotide probe. The probed blot was washed for ten minutes in 2 x SSC buffer and thirty minutes in 1 x SSC buffer before rinsing with maleic acid buffer + 0.3% tween 20 and blocking for a further thirty minutes at room temperature in 1% blocking reagent (Boerhinger Mannheim Cat. No. 1 096 176) dissolved in 20 mls maleic acid buffer + 0.3% tween 20. Blots were then conjugated for thirty minutes at room temperature with 1µl commercially obtained alkaline phosphatase linked sheep Fab' fragments to digoxigenin (Anti-dig-AP; Boerhinger Mannheim Cat. No. 1 093 274). Following two further fifteen minute washes in maleic

acid + 0.3% Tween 20 and a rinse in buffer 3, the blots were incubated for five minutes with 0.1% CDP-star™ chemiluminescent substrate (Boerhinger Mannheim Cat. No. 1 685 627) dissolved in buffer 3. The blots were finally transferred to a radiographic film cassette containing Hyperfilm™-ECL high performance luminescence film (Amersham International, Cat. No. RPN 2103) and exposed for five minutes. Dark bands on the processed radiographic films indicated where dig-labelled probe hybridisation had occurred with the cDNA in the PCR amplified samples. The blots could also be stripped of the probes to allow re-hybridization with another probe or the same probe using different hybridization conditions (see below). This was achieved by giving the blots two fifteen minute washes at 40 °C in 0.2M NaOH + 0.1% SDS followed by three five minute washes at 40 °C in 2 xSSC + 0.1% SDS. The blots were then re-exposed for 30 minutes to ensure that all dig-labelled probe had been removed.

2.7.10 High and low stringency hybridization conditions for whole enzyme cDNA probing

In addition to using oligonucleotide cDNA probes which were generally 25-30 base pairs (bp) in length, the latter stages of the sequencing study also involved hybridizing blotted samples with much larger (> 500 bp) whole enzyme cDNA probes. Moreover, the hybridizations had to be carried out at differing temperatures and washing stringencies to allow selective binding of the probes to blotted samples which demonstrated only the highest degrees of identity with the probe. For 'low stringency' hybridizations which allowed less specific probe binding, the probe hybridization step was carried out at 50 °C for three hours followed by a ten minutes 2 x SSC and thirty minute 0.5 x SSC wash. For 'high stringency' hybridizations which allowed more selective probe binding, the hybridization step was carried out at 65 °C for three hours followed by a ten minute 2 x SSC and thirty minute 0.2 x SSC wash.

2.7.11 Cloning of mast cell protease-specific cDNA fragments

Secondary PCR products positive for mast cell protease cDNA were inserted between the P lac promoter and lacZα (β-galactosidase) gene of an Invitrogen 3.9kb pCR™2.1 plasmid vector which also contained genes for ampicillin and kanamycin resistance (Fig. 2.2). The ligation reaction was carried out overnight at 15 °C according to the manufacturer's instructions (TA cloning kit; Invitrogen Cat. No. 2331). The ligated plasmids were transformed into 'One Shot' competent Invitrogen *E. coli* cells (TA cloning kit; Invitrogen Cat. No. 2331) which were then plated out onto Luria Broth (LB) agar plates containing 50μg/ml kanamycin (Sigma Cat. No. K 0879) and 10μg/ml x-galactopyranoside (Appendix A). The plates were incubated overnight at 37 °C and stored for at least two hours at 4 °C. They were then screened for the presence of white colonies which indicated the presence of transformed *E. coli* containing ligated plasmids, due to the disruption of the plasmid derived β-galactosidase expression. These colonies were picked off the plates and inoculated into sterile universals containing LB media and 1μg/ml ampicillin (Appendix A) for further selection and cloning overnight at 37 °C.

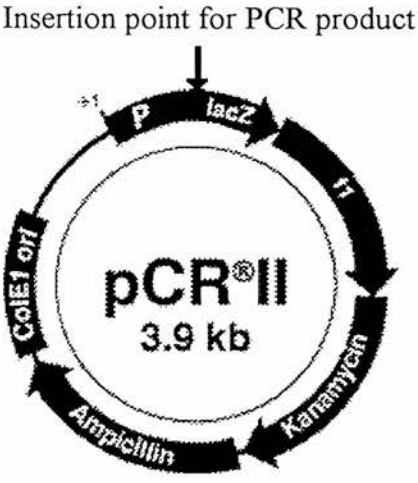


Fig. 2.2 Map of the Invitrogen pCR®II plasmid showing the genes for ampicillin and kanamycin resistance as well as the insertion point for PCR products between the Plac promoter and the lacZα gene

2.7.12 Plasmid DNA purification

Overnight selected *E. coli* suspension (3mls) was harvested from the LB media universals and processed using a commercial kit (Qiaprep Mini-prep® plasmid DNA purification system; Qiagen Cat. No. 27104) according to the manufacturer's instructions.

2.7.13 Restriction enzyme digestion of the plasmid inserts

Aliquots (1 µl) of the purified plasmid DNA preparations were incubated for one hour at 37 °C with 0.01µg /ml EcoRI (Boerhinger Mannheim Cat. No. 703 737), 0.01µg/ml NotI (Boerhinger Mannheim Cat. No. 1 014 706), or no restriction enzyme (uncut). pCR™2.1 incorporates two cleavage sites for EcoRI positioned at either end of the ligated DNA insert and a single NotI site positioned 3' to the DNA insert. Incubation with these enzymes therefore resulted in either the complete removal of the DNA insert from the plasmid (EcoRI) or linearisation of the plasmid (NotI). The

restriction enzyme digestion products were separated on an agarose gel and blotted with a mast cell protease-specific dig-labelled probe as outlined above.

2.7.14 DNA sequence analysis of plasmid inserts

Plasmid preparations containing probe positive inserts were checked for their DNA content and purity on a Beckman DU-650 spectrophotometer by measuring the OD_{260nm} and OD_{260nm}/OD_{280nm} ratio for samples diluted 1:50 in ddH_2O. 20µl sample aliquots containing > 200µg/ml DNA with an OD_{260nm}/OD_{280nm} value > 1.5 were sent to the Department of Biological Sciences, Durham University, England for sequence analysis. Samples were sequenced in both the forward and reverse directions and the results compared, to minimise the possibility of sequencing errors due to non-identified nucleotides.

2.8 Histochemical and immunohistochemical staining of tissues and cytosmears

2.8.1 Tissue fixation, embedding and cutting

Tissues were routinely fixed for six hours in 4% paraformaldehyde in PBS or modified Bouin's fixative (Appendix A) at room temperature (Newlands, Huntley and Miller 1984). Thereafter, they were transferred to 70% ethanol and stored at 4 ^{o}C until they were embedded in paraffin wax. Serial 4µm sections were cut from the embedded tissue blocks using a microtome and attached to 0.1% poly-l-lysine (Sigma Cat. No. P 8920) treated glass slides by heating for two hours at 60 ^{o}C in an oven.

2.8.2 Cytosmear preparations

Cytosmear preparations of goat BMMC and isolated goat MMC and GL were made using a Shandon Cytospin 2 cytocentrifuge (Shandon Southern Products Ltd.). Aliquots (200µl) of cell suspension containing 2 x 10^5 to 1 x 10^6 cells/ml were

centrifuged for five minutes at 800 rpm. Cytosmears were air dried for fixation or staining. Cytosmears for immunohistochemical staining were fixed in 4% paraformaldehyde in PBS for one hour at 40 ^{o}C, rinsed in tap water and stored in 70% ethanol at 4 ^{o}C prior to use.

2.8.3 Toluidine blue staining for mast cells

This was modified from the original technique of Enerback (1966a). Slides containing the paraffin embedded tissue sections were dewaxed by incubating in two changes of xylene for ten minutes and passing through a graded alcohol series (Appendix A). The slides were then placed in 0.5% toluidine blue pH 0.5 (Appendix A) overnight. Stained slides were washed for ten minutes in running tap water before dehydrating through a graded alcohol series (Appendix A) and mounting in poly-vinyl pyrrolidone (PVP) (Tissuetek II, Naperville, USA).

2.8.4 Carbol chromotrope staining of tissues for GL and eosinophils

Dewaxed tissue sections were pre-stained for one minute in haematoxylin (Appendix A) and washed for ten minutes in running tap water. They were then stained for one hour in Lendrum's carbol chromotrope (Lendrum 1944) (AppendixA) at room temperature before washing for ten minutes in running tap water, dehydrating and mounting in PVP.

2.8.5 Carboxybenzoyl-L-lysine thiobenzyl (BLT-) esterase staining of tissues

This was carried out using a modification of the technique of Kramer, Fruth, Simon and Simon (1989) for the detection of T cell-derived serine protease -1 granzymes. Dewaxed sections were incubated for fifteen minutes at 37 ^{o}C in a solution of carboxybenzoyl-L-lysine thiobenzyl ester and fast blue BB salt pH 8.2

(Appendix A). They were washed for five minutes in running water and mounted in Apathy's water soluble mountant (Raymond-Lamb, London, Cat. No. AE004-A).

2.8.6 Leishman's staining of cytosmears

Air-dried, unfixed cytosmears were laid out on a staining rack and flooded with 1 ml Leishman's staining solution (Appendix A) for two minutes. 1ml of tap water was then added and the mixture left for a further five minutes. After rinsing thoroughly in tap water, the slides were air dried and mounted in PVP.

2.8.7 Immunohistochemistry protocol for detecting mast cell proteases in tissue sections and cytosmears

Dewaxed tissue sections and fixed cytosmears stored in 70% ethanol were treated with 100% methanol + 0.5% H_2O_2 (Appendix A) for thirty minutes at room temperature to block endogenous peroxidase activity before washing for fifteen minutes in three changes of PBS . All slides were washed for ten minutes in two changes of PBS and blocked for thirty minutes at room temperature with 4% bovine serum albumin dissolved in PBS (BSA/PBS). They were then incubated overnight in a humidity chamber at 4 ^{o}C with primary antibody diluted in 4% BSA/PBS (1:200 for polyclonal rabbit anti-GMCP IgG or 1:500 for polyclonal rabbit anti-SMCP and polyclonal rabbit anti-human tryptase) or neat hybridoma cell supernatant. After washing for 15 minutes in three changes of PBS they were incubated for 1 hour at room temperature with a horseradish peroxidase (HRPO) or biotin conjugated secondary antibody diluted 1:500 in 4%BSA/PBS. Staining procedures involving the use of a biotinylated secondary antibody required a further PBS wash and incubation step for another thirty minutes at room temperature with streptavidin peroxidase (S-POD) (Boerhinger Mannheim Cat. No. 1089 153) diluted 1: 4000 in 4% BSA/PBS. Peroxidase-binding to mast cell proteases was visualised by washing the slides for a further ten minutes in PBS and reacting with 3'3'-diaminobenzidene and H_2O_2 (DAB)

substrate solution (Sigma Fast™ DAB tablets; Sigma Cat. No. D 4293) for five minutes at room temperature. Finally, the slides were counterstained for one minute in haematoxylin (Appendix A) and washed for five minutes in running tap water before dehydrating and mounting in PVP.

2.8.8 Dual immunofluorescence protocol for detecting mast cell proteases in tissue sections.

This was carried out on modified Bouin fixed sections using a similar protocol to that described for peroxidase staining of 4% paraformaldehyde/PBS fixed tissues with the following modifications. Following treatment for endogenous peroxidases, the sections were washed in PBS for ten minutes and blocked with DAB + H_2O_2 (Sigma Fast™ DAB tablets; Sigma Cat. No. D 4293) for five minutes to reduce auto-fluorescence. They were initially incubated with mouse monoclonal antibodies to GMCP diluted 1:200, followed by a sheep anti-mouse Ig antibody conjugated to fluoroscein isothiocyanate (sheep anti-mouse Ig FITC, Moredun batch no. 99/91) diluted 1: 50 in 4% BSA/PBS. They were then incubated with rabbit polyclonal antibody to SMCP diluted 1:500 followed by a goat anti-rabbit IgG antibody conjugated to tetra-rhodamine isothiocyanate (goat anti-rabbit IgG TRITC, Dakopatt, Denmark Cat. No. R 0156) again diluted 1: 50 in 4% BSA/PBS. After washing for fifteen minutes in three changes of PBS, the slides were mounted, without dehydrating, in Citifluor® non-fluorescent mountant (Citifluor Ltd., London) for viewing on an Ortholux II U.V microscope (Leitz, Germany) fitted with interchangeable purple and green excitation filters for viewing the FITC and TRITC staining cells respectively. Control sections were incubated with normal rabbit serum diluted 1:500 in place of anti-SMCP and murine ascites fluid diluted 1:500 containing an unrelated monoclonal antibody (VPM-12) to the NS2-3 (non-structural) pestivirus polypeptide present in Border Disease Virus (Entrican, Dand and Nettleton 1995) developed at MRI and kindly provided by Dr. G. Entrican and Mr. D. Deane. Control

sections treated with DAB alone were also examined for the presence of autofluorescence.

2.8.9 Counting protocols for tissue sections and cytosmears

Stained cells in the tissue sections were counted under a x 10 eye-piece containing a calibrated graticule and x 40 objective lens viewing an area of 0.08 mm^2. In the abomasal sections, counts were made systematically from the mucosal surface to the muscularis layer on a minimum of 25 graticule fields per tissue segment (Fig. 2.3a) and expressed as the mean number of mast cells per 0.2mm^2. Counts in the jejunal tissues were made on a minimum of 20 villus crypt units (VCU) (Fig 2.3b) and expressed as the mean numbers of mast cells per VCU. For both tissues, duplicate counts were made on a minimum of two separate tissue segments trimmed from separate areas of the fixed tissue. When counting cytosmears, a minimum of 400 cells were randomly counted per slide with the final result expressed as the percentage of mast cells relative to the total cell population present in the cytosmear.

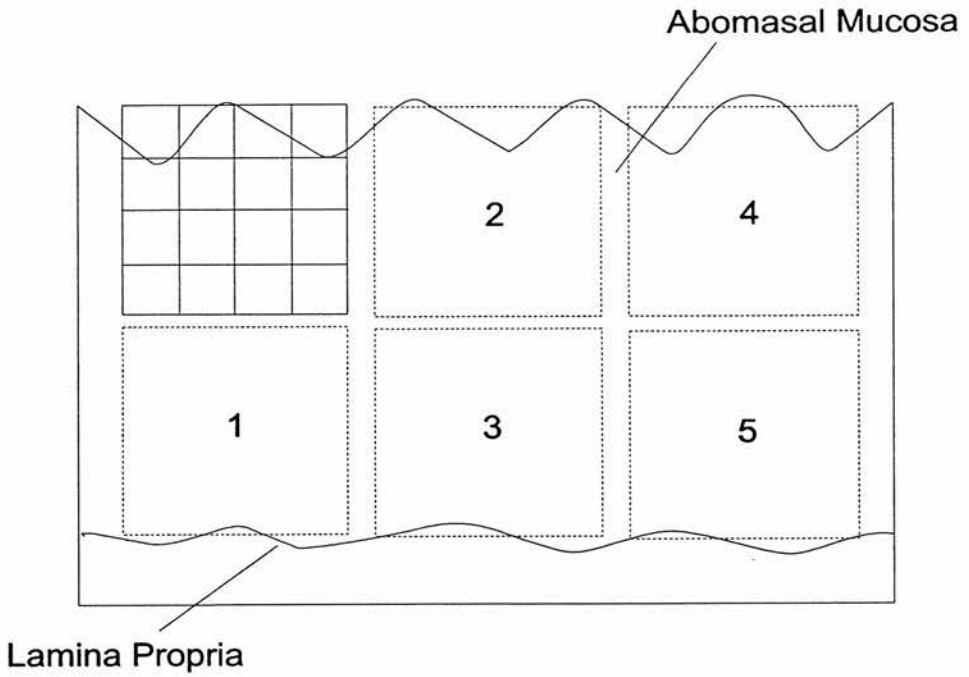


Fig. 2.3a Counting protocol for abomasal tissues showing approximate graticule area and the field sequence for five areas to be counted.

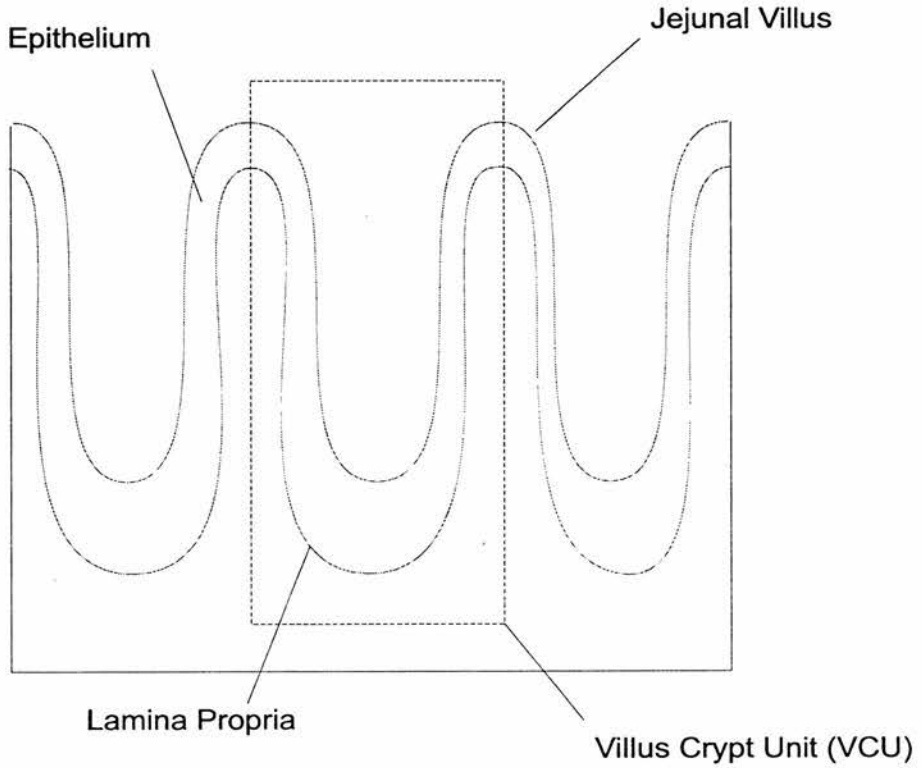


Fig. 2.3b Counting protocol for jejunal tissues showing one villus crypt unit (VCU)

2.9 Isolation of goat BMMC and GL

2.9.1 Trickle infection of animals

Animals used for the isolation of MMC and GL were all more than three years old and had been previously exposed to natural infection whilst maintained on pasture. Three animals were given weekly doses of 2000 *T.vitrinus* and 2000 *T. circumcincta* L_3 (kindly provided by Dr. F. Jackson) *per os* for 8 weeks, and euthanased 10 days after the final dose. These animals were also used to provide tissues for the GMCP tissue distribution experiment described in Chapter 4.

2.9.2 Isolation of MMC and GL

This procedure was carried out according to the method of Jones, Huntley and Emery (1992). Immediately post mortem, 50cm sections of jejunum were harvested from an area commencing 1 metre distal to the pylorus, opened longitudinally, rinsed and stored in PBS. In the laboratory, the jejunal segments were transferred to 100mls isotonic Hanks balanced salt solution (HBSS) (Gibco BRL, Life technologies Ltd. Cat. No. 041-04020/1M) pre-warmed to 37 ^{o}C, and 40mg Dispase II neutral protease (Boerhinger Mannheim Cat. No. 165 189) was added. The mixture was incubated for sixty minutes at 37 ^{o}C with agitation on a rotary shaker. The enzyme reaction was inhibited by the addition of 2mls foetal calf serum (FCS) (Advanced Protein Products Ltd. Cat. No. AS-302-50) and 2000 iu heparin (tissue culture grade 1A, Sigma Cat. No. H3149) to reduce cell aggregation. The remnants of the jejunal tissues were then removed and the remaining cell suspension was washed twice by centrifuging for five minutes at 200 x g and re-suspended in HBSS + 2% FCS + P/S + heparin (Appendix A; tissue culture media). A viable cell count was performed by diluting the cells 1:10 in 0.1% nigrosin/eosin dye and counting duplicate samples on a Cristalite™ modified

Neubauer haematocytometer (Hawksley, England). Sufficient cells were removed to provide 4 x 10^7 cells for each gradient as well as cytosmears for Leishman and antibody staining. Discontinuous Percoll™ (Pharmacia Cat. No. 17-0891-01) density gradients were constructed in clear polypropylene tubes as outlined in Appendix A. Cells removed for gradient purification were centrifuged and re-suspended in sufficient 55% isotonic Percoll (IP) to provide 2mls suspension for each gradient. The suspension was carefully layered on top of the 65% IP portion of the gradients which were then centrifuged at 600 x g for 30 minutes in a swing-out rotor with no brake (Beckman, model TJ-6 centrifuge) at 4 ^{o}C.

Following centrifugation, cells at the 65% / 80% inter-phase were harvested, pooled and diluted with at least 4 volumes of HBSS + 2% FCS + P/S + heparin. The purified cells were washed twice and re-suspended in 10mls Earles solution + 2% FCS (Appendix A) for a viability cell count and cytosmear samples. Cells deemed to be sufficiently pure by Leishman staining (60-70% MMC and GL) were then used for release studies and electron microscopy.

2.9.3 Cell fixation for electron microscopy

Harvested isolated MMC/GL or BMMC (see below) were transferred to eppendorffs and centrifuged at 200 x g for five minutes at 1200 rpm in a microcentrifuge (Costar Model 10, Sigma Cat. No. M3033), to produce a pellet containing 1 x 10^6 cells. The supernatant was removed and the cell pellet resuspended in 1ml of freshly made up fixative containing 3% gluteraldehyde and 0.1M sodium cacodylate. The cells were fixed for 24 hours at 4 ^{o}C before delivering to Mr. S. Mitchell at the Electron Microscope Unit of the Royal (Dick) School of Veterinary Studies for processing. Following post-fixation in 1% osmium tetroxide in 0.1M sodium cacodylate, the cells were embedded in Araldite™ overnight before cutting and mounting onto 200 mesh copper grids. The grids, containing approximately 5 - 20

cells per square, were then viewed using a Phillips 400® transmission electron microscope and the best cells selected for photographing.

2.10 *In vitro* growth of goat bone marrow mast cells

After the harvesting of the sternal bone marrow (see 2.10.1), all procedures were carried out under aseptic conditions in a tissue culture hood (ICN Flow Gelaire® Class II BSB48, ICN Pharmaceuticals Ltd.) using sterile media and equipment.

2.10.1 Harvesting of sternal bone marrow cells

This procedure was carried out using a modification of the technique of Haig *et al.* (1988) for the collection of ovine sternal bone marrow. Animals were killed by exsanguination after captive bolt stunning. Immediately post mortem the sternum was removed and split longitudinally from the xiphoid cartilage to the manubrium with a surgical saw. The marrow was extracted by squeezing the sternum in a vice and collecting the exudate from the cut surface with a sterile scalpel blade. The extracted marrow was resuspended in 20 ml of sterile Hanks balanced salt solution (HBSS) containing 5% foetal calf serum (FCS), 100 U/ml penicillin, 50 µg/ml streptomycin (P/S) and 20 U/ml heparin (Appendix A) for transport to MRI. In the laboratory, the cell suspension was removed from below the fat layer, filtered through two thicknesses of sterile lens tissue to remove fat and debris and washed twice in fresh HBSS + 5% FCS + P/S + heparin by centrifugation at 200 x g in a Beckman Model TJ-6 centrifuge at 4 ^{o}C for five minutes before re-suspending each preparation in 24ml of the same medium. 4ml aliquots of the washed cell suspension were carefully overlaid onto 6 mls of Lymphoprep^{tm} (Bayer, GmBH) for centrifugation at 400 x g for thirty minutes at room temperature. The resulting inter-phase cells were harvested, pooled and diluted in at least four volumes of HBSS + 5%FCS + P/S + heparin and washed three times as above, to remove any residual Lymphoprep. After the final wash, the

cells were resuspended in 20 mls of sterile Iscoves's modified Dulbecco's medium (IMDM) containing 5 x 10^{-5}M 2-mercaptoethanol (2-ME), 10% FCS and P/S (Appendix A). A viable cell count was performed before adjusting the cell density to 2 or 3 x 10^5 cells/ml in IMDM + 2-ME +10%FCS + P/S.

2.10.2 Feeding and incubation of cultures

The adjusted cell suspensions were aliquoted into either 24 well plates (24 well culture cluster, Costar Cat. No. 3524) or 75 - 225 cm^3 canted neck tissue culture flasks (Corning Cat. No. 25110-75). The recombinant cytokines, ovine interleukin-3 (rOvIL-3; Moredun batch no.1H2 3/2/10/TF/1/C/9/2) and/or ovine stem cell factor (rOvSCF; Moredun batch no.3/2/7/2/TF/2/A/3/12/3) were added in the appropriate combination and concentration for the current experiment (Chapter 7) then the cells incubated in an atmosphere of 5% CO_2 in air at 37 ^oC in a tissue culture incubator (ICN Flow automatic CO_2 Incubator Model 160, ICN Pharmaceuticals). Cultures in 24 well plates were fed with fresh cytokine(s) every 48 - 72 hours by removing 500µl of cell-free medium from each well and replacing it with 500µl of IMDM + 10%FCS + P/S + 2-ME incorporating the appropriate dilution of cytokine. Flask cultures were also fed at the same time interval by adding the appropriate volume of cytokine supernatant to give the correct final dilution. Every seven days, the cells were centrifuged at 200 x g for five minutes and resuspended in fresh IMDM + 2-ME +10%FCS + P/S. Throughout the culture period, the cells were monitored daily under a Leica DM 1L inverted microscope (Leica microscopy Cat. No. 520802/152192) and by microscopic examination of Leishman's stained cytosmear preparations.

2.10.3 Cell harvesting

24 well plates

After gentle aspiration with a sterile wide bore Pasteur pipette, 250µl of cell suspension from each well was removed for viable cell count and cytosmear preparations. The remaining cell suspension volume was measured before being transferred to 1.5ml eppendorff tubes and centrifuged at 200 x g for 5 minutes at 1200 rpm in a microcentrifuge (Costar, Model 10, Sigma Cat. No. M3033). The resulting supernatant was carefully removed and stored along with the cell pellet at -20 ^{o}C pending further analysis. The results of the cell count were corrected for viability and total cell suspension volume, with the final results expressed as total cell numbers per well x 10^{5}.

Tissue culture flasks

After gentle agitation, 500 -1000µl aliquots of cell suspension were removed for a cell count and cytosmear preparations. Sufficient cell suspension was then removed to provide a cell pellet containing 1 x $10^{6,}$ cells which was collected and stored as described above.

2.10.4 Extraction of cell pellets for mediator assays

This technique was modified from that of Huntley (1991) for the extraction of ovine BMMC cell pellets. Granule-associated mediators were solubilised from the harvested cell pellets by lysing the cells in 200µl of 20mM Tris + 1.5M NaCl pH 7.5 (extraction buffer, Appendix A) . The suspension was sonicated for sixty seconds in a Polaron sonicator (Polaron Ltd. England) and rapidly freeze-thawed three times. The resulting extract was centrifuged at 10, 000 rpm for five minutes in a microcentrifuge (Costar, model 10) to remove residual cell debris.

2.11 Secretogogue and worm antigen-mediated release of goat bone marrow mast cells (BMMC) and mucosal mast cells (MMC)

2.11.1 Secretogogue-mediated cell release protocol

Aliquots of purified MMC and cultured BMMC harvested between days 16 and 22 of culture were washed twice by centrifuging at 200 x g and re-suspending in 1 x Earle's medium containing Mg^{2+} and Ca^{2+} without phenol red + 2% FCS (Appendix A). A cell count was performed and the cell suspension adjusted to 0.5-1 x 10^7 viable cells per ml. Aliquots (100 µl) of the cell suspension each containing 0.5-1 x 10^6 cells, were transferred to 1.5ml eppendorff tubes containing 100µl of the appropriate secretogogue diluted in 1 x Earles medium + 2% FCS to twice the final reaction concentration. The cell suspension/secretogogue mixture was then incubated at 37 ^oC in a shaker water bath (Grant Instruments model No. 55-40D, Cambridge) for sixty minutes. At the end of the incubation period, the samples were centrifuged for three minutes at 200 x g and the supernatants carefully collected for storage at -20 ^oC. All samples were tested in duplicate with matched negative controls incubated with 1 x Earles + 2% FCS alone in place of diluted secretogogue. Positive controls to measure total cell mediator content consisted of duplicate 100 µl cell suspension aliquots harvested prior to the incubation step by centrifugation at 200 x g without the addition of secretogogue or diluent. The resulting pellets were lysed as described previously for harvested BMMC and the extracts stored at -20 ^oC.

2.11.2 Lymph and serum sensitisation of goat BMMC

Abomasal afferent gastric lymph and serum samples were kindly provided by Dr. F. Jackson and Dr D.M. Patterson. They were were tested for the presence of IgE by dot blotting and western blotting reduced samples using a rat monoclonal antibody supernate to a fragment of recombinant ovine IgE (1E7, anti-ovine ε-chain; Kooyman, van Kooten, Huntley, Mackellar, Cornelissen and Schallig, 1997) as described below

(2.13). Samples containing the highest concentrations of IgE, along with heat-inactivated controls which had been incubated at 56 ^{o}C for two hours, were used to sensitise caprine BMMC for FcεRI mediated granule release in the presence of *Telodorsagia circumcincta* and *Haemonchus contortus* nematode antigen preparations. BMMC harvested between days 16 and 22 were washed twice in 1 x Earle's medium without FCS, re-suspended in 2mls of lymph or serum diluted 1:4 in 1 x Earles, and incubated for forty five minutes at 37 ^{o}C in a shaker water bath. After washing three times in 1 x Earles, sufficient cells were removed for the antigen release studies and the remainder retained for cytosmear preparations or placed on ice for immunofluorescent surface-bound immunoglobulin staining.

2.11.3 Immunofluorescent staining of goat MMC and sensitised goat BMMC for surface immunoglobulin

Aliquots of isolated MMC, serum sensitised BMMC and heat-inactivated serum sensitised BMMC, containing 5 x 10^{6} cells in 1 x Earles were maintained on ice (cells were maintained on ice throughout the procedure to minimise capping and internalisation of surface bound immunoglobulin). Murine ascites fluid containing monoclonal antibody to ovine immunoglobulin light chain (Bird, Jones, Allen, Donachie, Huntley, McConnell and Hopkins, 1995) (VPM-8, kindly provided by Mr. D. Deane) was added to produce a final antibody dilution of 1: 500. The cells were mixed by inverting 6 times before incubating for 30 minutes with occasional agitation. The cells were then washed three times in ice-cold 1 x Earles before adding affinity-purified polyclonal sheep anti-mouse IgG FITC (Dakopatt, Denmark Cat. No. F 0261) to produce a final antibody concentration of 1:50. The cells were again mixed and incubated for a further sixty minutes before washing three times in ice-cold 1x Earles. Cytosmear preparations were made of the stained cells and the slides mounted in citiofluor® for viewing under an ortholux II U.V. microscope (Leitz, Germany) as described above for immunofluorescent antibody stained tissue sections. Controls

consisted of replacing VPM-8 primary antibody with a murine monoclonal antibody directed against Border Disease virus non-structural proteins (VPM-12) and, in the case of BMMC, by staining for surface immunoglobulin on cells sensitised with IgE positive goat serum which had been heat inactivated at 56 ^{o}C for 2 hours.

2.11.4 Worm antigen preparations

These were kindly provided by Dr. D. Knox and Dr. J. Huntley, having been prepared from nematodes provided by Dr. F Jackson. Six preparations were routinely used for testing; these consisted of *T. circumcincta* L_3, L_3 PBS soluble whole worm homogenate antigen (WWA) and excretory/secretory antigen (ES) proteins, and *H. contortus* L_5 WWA and ES proteins. Unfortunately, due to limited stocks, equivalent *H. contortus* L_3 preparations were unavailable. Briefly, WWA proteins were prepared by homogenising either 5000/ml L_3 or 60-100/ml adult parasites in PBS with sterile quartz sand (Sigma Cat. No. S 9887) in a hand held glass homogeniser (Pyrex). The homogenised samples were then centrifuged at 600 x g for five minutes and the supernatant harvested. Supernatant batches were tested for their protein content by measuring 1.3 x their OD_{260nm} on a Beckman DU-650 spectrophotometer then aliquoted and stored at -20 ^{o}C. For ES proteins, the same numbers of L_3 or adults were maintained, with constant gentle agitation, for sixteen hours at 37 ^{o}C in sterile RPMI 1640 medium. Parasite viability at the end of the culture period was confirmed on the basis of structural integrity and motility. The culture supernatant was concentrated twenty fold using Centricon-10 filters (Amicon Ltd., Stonehouse, U.K.) and centrifugation at 1500 x g (4 ^{o}C) for one hour in a MSE Mistral 6L centrifuge. The protein content of the concentrated samples was assessed as for the WWA preparations for aliquoting and storing at -20 ^{o}C. Freshly thawed aliquots were used for each experiment.

2.11.5 Worm antigen-mediated release protocol

This was carried out as for the secretogogue mediated release protocol described above, however the BMMC were initially sensitised with IgE-positive or heat inactivated goat lymph and serum from animals challenged with *T. circumcincta*. All worm antigen preparations were tested at a final reaction concentration of 50µg/ml and incubated with the cells for 30 minutes at 37 ^{o}C. The final results were corrected for background spontaneous release by subtracting the percentage mediator release in supernatants from paired control samples undergoing sensitisation only without exposure to the nematode antigen preparations.

2.11.6 Calculation of percentage mediator release from stimulated BMMC and MMC

After testing the released cell supernatant samples for GMCP, β-hexosaminidase and aryl-sulphatase content as described below, the % secretogogue or nematode antigen mediated release from the cells was calculated as follows:

$$\% \text{ release} = \frac{([\text{mediator}] \text{ in supernatant} - [\text{mediator}] \text{ in diluent only control} - [\text{mediator}] \text{ in the releasing agent})}{[\text{mediator}] \text{ in the harvested cell pellet}} \times 100$$

2.12 Enzyme assays for goat bone marrow mast cells

2.12.1 SMCP and GMCP enzyme linked immunosorbent assays (ELISAs)

ELISAs for SMCP and GMCP utilised the double antibody sandwich design for the detection of SMCP developed by Huntley *et al.*(1987). Both assays consisted of homologous polyclonal capture antibodies and homologous rodent or murine monoclonal detection antibodies which had been conjugated to biotin (Fig 2.4). All

antibodies used in the SMCP ELISAs had been developed previously by Dr. J. Huntley and A. MacKellar, whilst the purified SMCP required for the assay standards was provided by Dr A. Pemberton. All reagents used for the GMCP ELISAs were developed specifically for this study using the techniques outlined above.

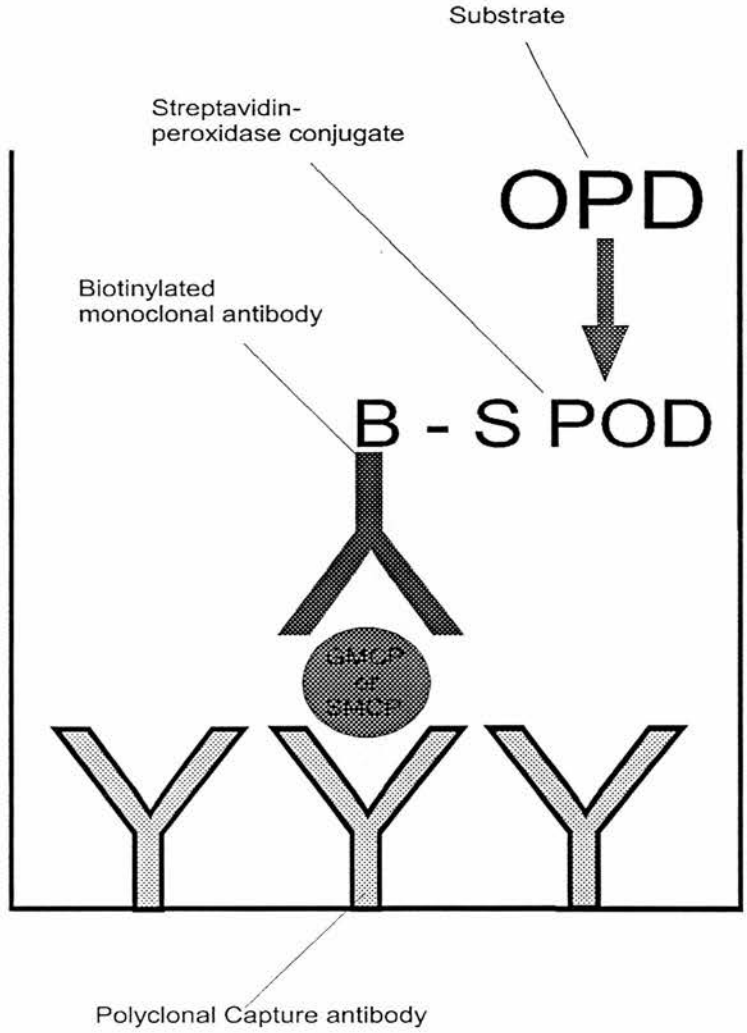


Fig 2.4 Double-antibody sandwich design for SMCP and GMCP ELISAs.

Polyclonal antibody stocks were diluted to 1µg/ml (SMCP affinity purified rabbit polyclonal antibody to SMCP, Batch No. A 95/1) or 10µg/ml (Protein A purified rabbit polyclonal IgG antibody to GMCP, Batch No. 177/95) in carbonate coating buffer pH 9.6 (Appendix A). 96 well microtitre ELISA plates (M129B, Dynatech Laboratories Ltd) were coated with 50µl per well of the diluted antibody

and incubated overnight at 4 ^{o}C. The plates were washed six times with PBS/0.05% Tween 20 (ELISA wash buffer, Appendix A) and loaded with duplicate 50µl aliquots of purified SMCP/GMCP standards (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 ng/ml) or samples diluted in PBS/0.05% Tween 20. The samples were assayed at a range of dilutions (1:10 to 1: 10 000 for tissue homogenates and undiluted to 1 : 50 for cell release supernatants), to ensure that at least one of the final sample optical density (OD) readings would lie within the standard curve OD range. The plates were incubated for sixty minutes at room temperature and washed six times with wash buffer before incubating for one hour with 50µl per well of biotinylated rat monoclonal antibody to SMCP (Batch No. 165/95) diluted 1:500 in PBS + 0.5% Tween 80 + 0.5M NaCl (AppendixA), or biotinylated murine monoclonal antibody to GMCP diluted 1: 200 in PBS + 0.5% Tween 80 + 0.5M NaCl. The plates were again washed six times and incubated for thirty minutes with 50µl per well of streptavidin peroxidase (Boerhinger Mannheim Cat. No. 1 089 153) diluted 1: 4000 in PBS + 0.5% Tween 80 + 0.5M NaCl. Following a further six washes, the colour reaction was developed by the addition of 50µl per well of orthophenylendiamine (OPD) substrate (Voller, Bidwell and Bartlett, 1979) in urea H_2O_2 buffer (SigmaFast™ OPD tablet sets, Sigma Cat No. P 9187). The colour reaction was terminated by the addition of 25µl 2.5M H_2SO_4 (Appendix A) and the plates read at OD_{492nm} in a Titertek® Multiskan MC Reader (Titretek® Flow Laboratories).

A standard curve based on the OD values given by the SMCP or GMCP standards was plotted using Dynatech data analysis software (Dynatech Laboratories Ltd), and the concentrations of protease in the samples under test calculated from the linear part of the curve.

2.12.2 β-Hexosaminidase Assays

Activity of the granule-associated acid hydrolase, β-hexosaminidase, was measured by hydrolysis of the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Cat. No. N9376); 1 unit of enzyme cleaving 1 μmole of substrate per hour at 37 ^{o}C (Schwartz, Austen and Wasserman, 1979). Duplicate 25μl samples of cell pellet extract or supernate were added to 50μl aliquots of 5mM substrate diluted in citrate buffer pH 4.5 (Appendix A) in a 96 well microtitre ELISA plate (M129A, Dynatech Laboratories Ltd). The mixture was incubated at 37 ^{o}C for forty five minutes then the reaction terminated with the addition of 100μl ice cold glycine NaOH buffer pH 10.7 (Appendix A).The resulting colour change was read at OD_{405nm} on a Titertek® Multiskan MC Reader (Titretek® Flow Laboratories) with the mean OD result being converted into approximate units of β-hexosaminidase activity using the equation:

β-Hexosaminidase activity (units) =

$$OD_{405nm}/(0.8 \times 18.8 \times 10^{3}) \times (TRV/TSV) \times 1.2735$$

(TRV = the total reaction volume and TSV = the total sample volume)

2.12.3 Aryl-sulphatase Assays

Aryl-sulphatase activity was measured by assessing the lytic activity of cell pellet extracts and supernatants against 6.255mM p-nitrocatechol sulphate (Sigma Cat. No. N 7251). Duplicate 20μl samples were added to 80μl of substrate dissolved in 0.2M sodium acetate pH 5.7 (Appendix A) in 96 well microtitre plates (M129A, Dynatech Laboratories Ltd) and incubated at 37 ^{o}C for sixty minutes before stopping the reaction with 100μl of 5M NaOH (Appendix A). Mean sample ODs were determined at 492 nm on a Titertek® Multiskan MC Reader (Titretek® Flow Laboratories) and compared with a linear 1.92-250 μg/ml standard curve of sulphatase-V (Sigma Cat. No. S 8629) diluted in 0.2M sodium acetate pH 5.7. The

standard curve was plotted using Dynatech data analysis software and concentration of aryl-sulphatase in the unknown samples calculated from the resulting linear equation.

2.13 Ovine IgE assays

A dot blot assay system using a a rodent monoclonal antibody supernatant to a recombinant fragment of ovine IgE ε chain (1E7 antibody (Kooyman *et al.*, 1997) kindly provided by Dr. H. Schallig, University of Utrecht and Dr. J. Huntley) was used to quantify the IgE concentration in serum and gastric lymph samples from goats undergoing parasitic challenge. The dot blot protocol was the same as that outlined above (2.5.1), apart from the sample preparation which required samples to be reduced by diluting 1:4 in reducing sample buffer (Appendix A) and heating to 100 ^{o}C for five minutes prior to blotting onto the NC membrane. This step was necessary to enable IgE detection by 1E7, suggesting that the monclonal antibody binds to a determinant normally hidden within the tertiary structure of native IgE (J. Huntley, personal communication). The samples were probed with the 1E7 antibody diluted 1 : 20 in PBS + 0.5% Tween 80 + 0.5M NaCl followed by a biotinylated rabbit anti rat IgG antibody conjugate (Dakopatt, Denmark Cat. No. E 0468) diluted 1: 500 in PBS + 0.5% Tween 80 + 0.5M NaCl. The average ODs of the DAB stained positive sample dots were determined by scanning the blots on a BioRad gel scanner (BioRad model No. 600) linked to a computer running BioRad 'Molecular Analyst' densitometry software (BioRad, Ohio, USA). The OD results for the unknown samples were then compared with a 0.875 - 7 μg/ml linear standard curve constructed from the recombinant ovine IgE ε chain protein used to produce the 1E7 monoclonal antibody (recIgE1-2, kindly provided by Dr. H.D.F. Schallig). The standard curve protein samples were double diluted in reducing sample buffer and heated to 100 ^{o}C for five minutes before blotting onto the NC membrane adjacent to the test samples. Final results for the unknown samples were expressed as μg total IgE per ml of serum.

Controls consisted of reduced serum samples taken from specific pathogen free (SPF) lambs as well as kids which had been maintained under worm-free conditions.

2.14 Data Analysis and Presentation

2.14.1 Statistical analyses

These were carried out using Excel™ version 5.0 (Microsoft Corporation, USA) for the calculation of group mean values, the standard deviation (SD) and linear regression analyses in Chapters 3,4 and 6, as well as all parametric analyses of grouped data in Chapters 3, 4, 7 and 8 using Student's two sample T-test. Minitab version 9.2 statistical software was used to analyse the distribution of $\log_{10}(x+1)$ transformed parasitological data (faecal egg counts and worm burdens) as well as untransformed cell count and serological data obtained from individuals in the experiment described in Chapter 6. The significance of grouped results in this experiment was tested using the non-parametric Mann-Whitney U test .

2.14.2 GMCP I and II cDNA and nominal amino acid sequence analyses

This was carried out by Telnet access via the Joint Academic Network (JANET) to the Seqnet computer based at Daresbury, England. Analyses were carried out using the Wisconsin Package© Version 8 (Genetics Computer Group, Madison, Wisconsin, USA)

2.14.3 Thesis presentation

This thesis was prepared using Microsoft Word™ version 2.0 (Microsoft Corporation, USA). Data charts were constructed using Excel™ version 5.0 (Microsoft Corporation, USA) and Microcal Origin™ scientific graphics package version 4.0 (Microcal Software, USA). Line diagrams were created using Corel Draw™ version 4.0 (Corel Corporation, Canada).

CHAPTER 3

THE ISOLATION AND PURIFICATION OF GOAT MAST CELL PROTEASE

3.1 Introduction

The aim of this chapter is to describe the isolation of a mast cell-specific protease from goat intestine, and to describe the development of immunospecific techniques for detecting this enzyme in host tissues. This will enable initial investigations to be made regarding the localisation, concentrations and catalytic properties of such an enzyme. Such findings will also allow more detailed assessments to be made of the comparative MMC responses shown by goats and sheep as a result of infection with gastro-intestinal nematodes. In addition, analysis of the substrate specificity of isolated GMCP will show whether it belongs to the newly emerging class of dual specific intestinal proteases recently isolated from other ruminants, including for example, bovine duodenase (Zamolodchikova *et al.*, 1995a and b) and SMCP (Pemberton *et al.*, 1997a and b).

3.2 Materials and methods

3.2.1 Animals

Thirty commercially reared Scottish cashmere goats (17 female, 13 male) aged between six months and four years were used. All had been exposed previously to repeated natural and artificial infections of *T. circumcincta* and *T. vitrinus* as outlined for the ADCM described in Chapter 2, section 3.2 (2.3.2)

3.2.2 Abomasal and jejunal tissue surveys for mast cell protease content

Samples (1g) of abomasal and jejunal tissues from each animal were homogenised for ELISA analysis as described in 2.3.3. Utilising the antigenic cross reactivity between SMCP and the presumptive GMCP (Huntley *et al.*,1995), these samples were assayed for potential GMCP content by SMCP ELISA as described previously (Huntley *et al.*, 1987), using the protocol outlined in 2.12.1. Tissues

containing the highest protease concentration were prepared for protease purification as described in 2.3.3.

3.2.3 Histochemical and immunohistochemical staining of mast cells and GL.

Sections (4μm) were prepared from 4% paraformaldehyde/PBS fixed abomasal and jejunal tissue from 6 does and 6 bucks randomly selected from the original group of 30 animals as well as 3 kids that were less than one year old which had been housed under worm-free conditions since birth (worm naive kids). In addition, 4 μm sections were also cut from 4% paraformaldehyde/PBS fixed, archived abomasal tissues taken from seven month old lambs which had been involved in an experiment one year previously (Coop *et al.*, 1995). During the experiment, these animals had undergone an ADCM regime similar to that described for the goats in 2.3.2. Tissues from three previously worm naive lambs which had received a single 50, 000 *T. circumcincta* L_3 challenge ten days before sacrifice (primary challenge lambs) were also included for comparison. Sections for histochemical analysis of MMC and GL were stained using toluidine blue and carbol chromotrope as described in 2.8.3 and 2.8.4, whilst sections for immunohistochemistry were incubated with polyclonal antibodies to GMCP and SMCP as described in 2.8.7. BLT-esterase staining and dual immunofluorescence studies incorporating murine monoclonal antibody to GMCP, and rabbit polyclonal antibody to SMCP, were also carried out on modified Bouin fixed goat tissues as described in 2.8.5 and 2.8.8

All other techniques employed for purification and characterization of GMCP, as well as the raising of antibodies and the construction of an ELISA for GMCP, were as described in the relevant sections in Chapter 2.

3.3 Results

3.3.1 ELISA survey of collected tissues

The mean (± SD) results obtained from the tissues of 30 goats tested with the SMCP ELISA were 0.945 ± 0.14μg/g for abomasal tissues and 2.37 ± 0.2μg/g for jejunal tissues. In the male goats there was significantly more protease present in the abomasal tissues (1.33 ± 0.24 μg/g) than in the females (0.64 ± 0.14 μg/g) ($p < 0.05$) although there was no significant difference between the sexes in the jejunal tissues (2.14 ± 0.47 μg/g and 2.67 ± 0.4 μg/g respectively). Overall, the goat jejunal tissues contained significantly higher amounts of protease ($p<0.001$) when compared to the abomasal tissues from either sex. As a result, the majority of GMCP purifications were carried out using jejunal tissue extracts.

3.3.2 Histochemical and BLT staining of fixed tissues

Fig. 3.1a and b and show cells staining positive with BLT substrate in modified Bouin fixed goat jejunal, and abomasal tissues. Fig. 3.1c and d show cells stained with toluidine blue in modified Bouin fixed abomasal, and 4% paraformaldehyde/PBS fixed jejunal tissues respectively. Fig. 3.1e also shows GL and eosinophils stained with carbol chromotrope in jejunal tissues fixed with 4% paraformaldehyde/PBS. Three of the cells in the abomasal section (Fig 3.1b) are identical to cells staining positive for toluidine blue in a paired serial section of the same tissue (Fig. 3.1c). BLT positive cells were also present in the sub-epithelial tissues of the jejunum (Fig. 3.1a) demonstrating an anatomical distribution similar to that seen in equivalent 4% paraformaldehyde/PBS tissues stained with toluidine blue (Fig. 3.1d). Faint BLT-positive intra-epithelial cells can also be seen in the jejunal tissues (Fig. 3.1a) which share a similar morphology and distribution to GL stained with carbol chromotrope on equivalent 4% paraformaldehyde fixed tissues (Fig. 3.1e).

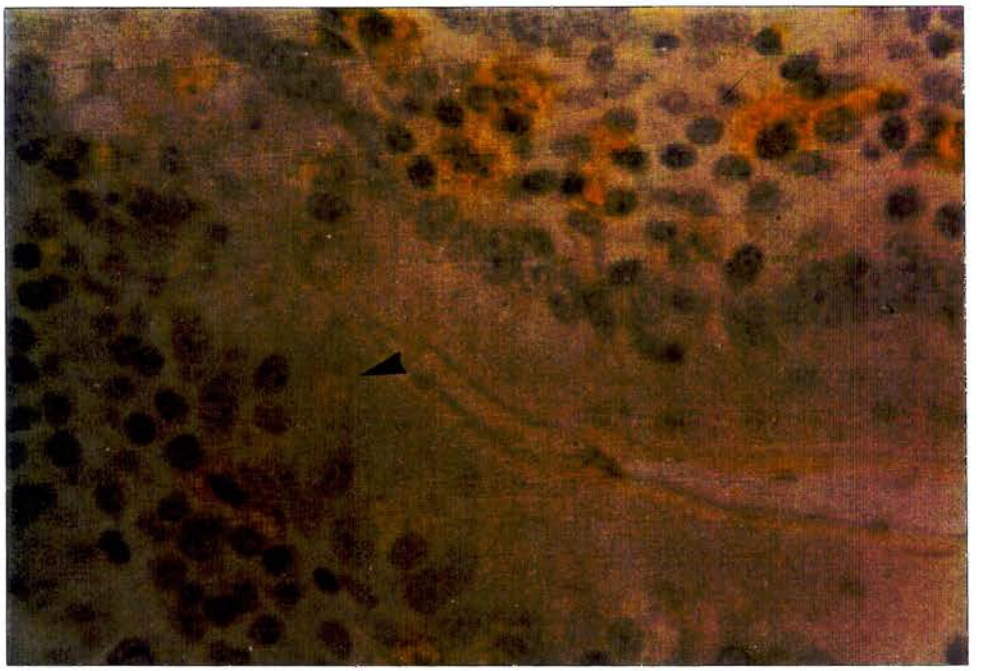


Fig. 3.1a Goat jejunal villus crypt stained with BLT, Note the faint positive staining intra-epithelial cells (arrow) and the sub-epithelial position of the other positive cells. Modified Bouin fixed tissue (x 400)

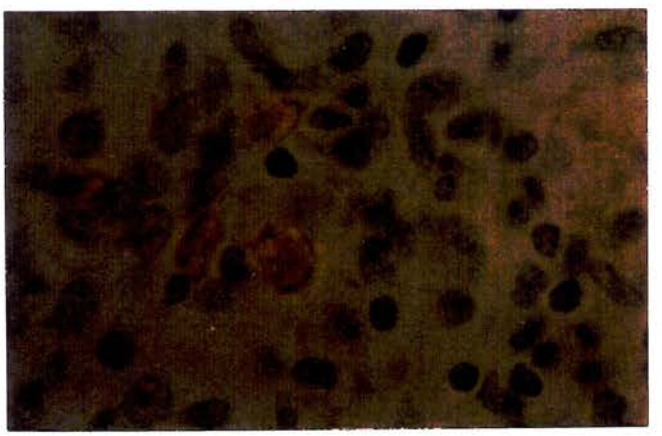


Fig. 3.1b Goat abomasal tissue stained with BLT, Note the triad of positive staining cells. Compare with the toluidine blue positive cells from the paired serial section in 3.1c. Modified Bouin fixed tissue (x 400).

Fig. 3.1c Goat abomasal tissue stained with toluidine blue. Compare the positive cells with the BLT stained cells in 3.1b. Modified Bouin fixed tissue (x 400).

Fig. 3.1d Goat jejunal villi stained with toluidine blue. Note the subepithelial position of the positive staining MMC. 4% Paraformaldehyde fixed tissue (x 100)

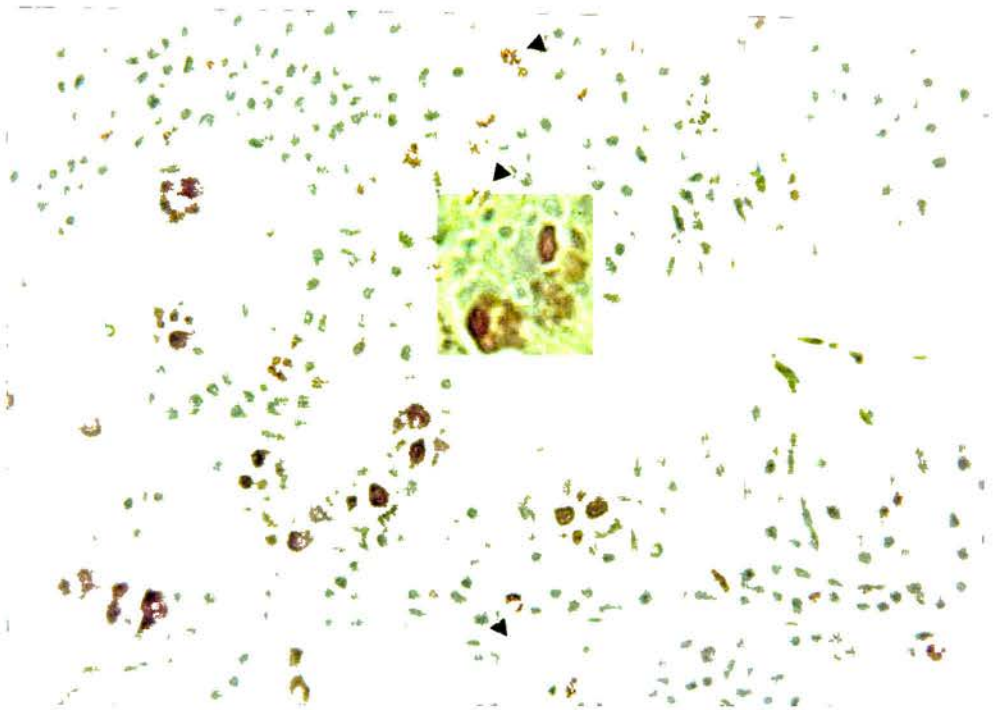


Fig. 3.1e Goat jejunal villus crypt stained with carbol chromotrope. Note the pink staining intra-epithelial GL, compare their position and morphology with the faint staining BLT positive cell in 3.1a. Note also the small, granular, darker staining eosinophils within the submucosal tissues (arrows). 4% Paraformaldehyde fixed tissue (x 250).

3.2.3 FPLC and immunoaffinity purification of GMCP

Table 3.1. shows the BLT substrate activity, SMCP ELISA and total protein content results for each stage of a typical separation starting with a 1.0M NaCl extract from 5g of jejunal tissue. Figure 3.2 shows typical elution OD profiles, measured at 280nm, for samples exiting CM-sepharose and MonoS FPLC columns after the application of an NaCl gradient.

Samples	BLT (ΔO.D./mg/ml)*	Total Protein (mg)	SMCP ELISA (μg)
1.5 M NaCl Homogenate for ELISA	ND	ND	6.17**
1.0 M NaCl Tissue extract	0.13	2.54	3.8
CM Sepharose Eluate	0.99	0.33	2.27
MonoS Eluate	18.66	ND***	1.51
% Yield of GMCP from homogenate			24.55

Table 3.1 BLT substrate activity, total protein content and SMCP ELISA determination for each step of a typical purification of 5g of goat jejunal tissue.ND = Not Done
* ΔO.D. = Change in sample optical density at 405nm after 5 minutes incubation at room temperature.**Result for 5g of jejunal tissue calculated from the SMCP ELISA result obtained from an equivalent 1g tissue aliquot homogenised in high (1.5M) NaCl buffer.*** = total protein content of sample below the assay sensitivity threshold of 12μg/ml.

Protein content and enzyme activity monitoring of the purification process (Fig. 3.2a and b) demonstrated that active fractions bound to the CM-sepharose column and could be eluted off in a broad peak at 0.4M NaCl (Fig. 3.2a). These eluted samples were diluted three volumes of 20mM Tris/HCl pH 7.5 and applied to the Mono S column, where the application of a stepped 0-0.5M NaCl buffer gradient produced a series of small peaks. Maximal substrate activity after five minutes incubation at room temperature occurred in peak fractions eluted at approximately 0.3-0.4 M NaCl (Fig.3.2b). The proteolytic activity of the eluted sample as measured

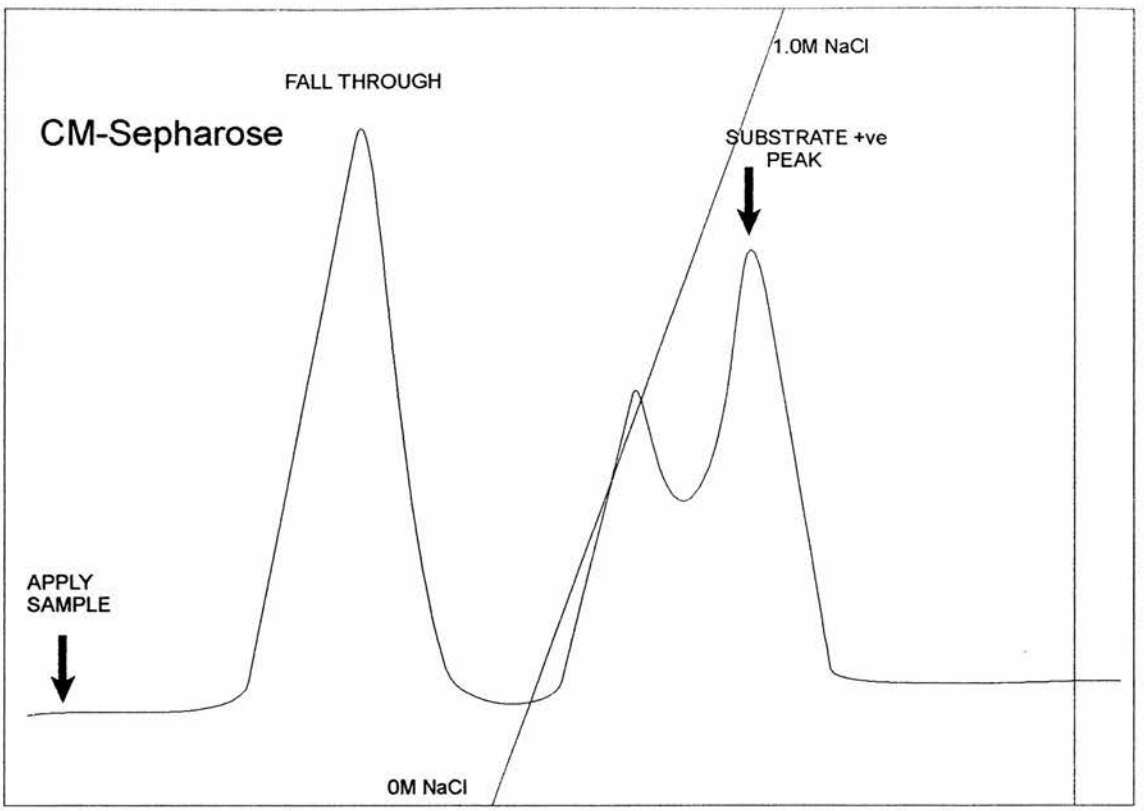


Fig.3.2a Schematic diagram of the OD_{280}nm elution profile obtained from the CM-sepharose column after applying a 0-1.0M NaCl gradient

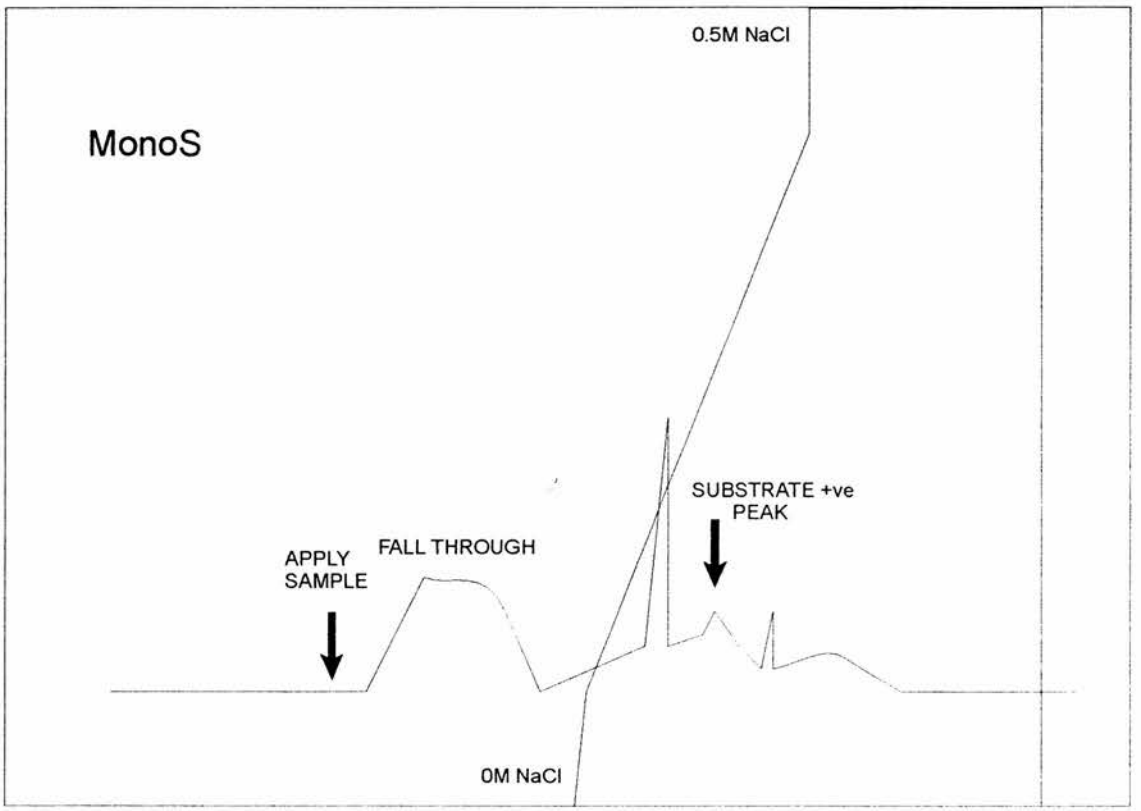


Fig.3.2b Schematic diagram of the OD_{280}nm elution profile obtained from the MonoS column after applying a 0-0.5M stepwise NaCl gradient

by the rate of reaction against BLT per mg of total sample protein (ΔOD/mg/ml) increased from 0.13 to 18.66 indicating increasing protease purity at each stage of the procedure (Table 3.1).

The final presumptive GMCP content of samples obtained, as assayed by SMCP ELISA, ranged from 1.02 μg to 7.635 μg depending on the initial GMCP content of the tissue used. This gave an estimated overall yield of GMCP recovered from the tissues of 20-30% (Table 3.1).

Immunoaffinity purification was initially much more successful, and achieved a greater than 80% recovery of the total tissue GMCP content (Fig. 3.3b lane 3). However, a significant reduction in column purification efficiency was demonstrated after only 2 or 3 purification runs. This may have been due to the presence of residual active proteases in the homogenate sample despite pre-incubation with Pefabloc^{tm} inhibitor or due to high affinity interactions between the protease and polyclonal anti-GMCP column antibody. Since the construction of each column required relatively large amounts of polyclonal antibody (5 mg of IgG per 1 ml of CNBr sepharose), which was of limited supply, this method of enzyme purification was used to provide samples for NH_2-terminal amino acid sequence analysis only.

3.2.4 Silver stain SDS-PAGE & Azocasein SDS-PAGE

SDS-PAGE of samples eluted from the MonoS column produced a separate band of approximately 29kD (Fig.3.3a, lane 5). This was identical in weight to SMCP (Fig 3.3a lane 6) and corresponded to a band of proteolytic activity found on the azocasein containing gels (Fig. 3.4 lane 3). The SMCP sample shown on the silver

stained SDS-PAGE gels appears as a doublet (Fig.3.3a lane 6 and Fig.3.3b lane 2) which may also be present in the immunoaffinity purified GMCP sample (Fig. 3.3b lane 3). However, in the case of SMCP, this is thought to be due to variable glycosylation of the enzyme since both bands share identical chromatographic and biochemical properties (A.Pemberton, personal communication) and can also be seen on Western blots probed with antibody to SMCP (not shown) and GMCP (Fig.3.5 lane 5; see 3.2.7 below). Non-reduced samples of isolated MMC/GL lysed in 20mM Tris HCl + 1.5 M NaCl (see Chapter 8) were separated on azocasein substrate gels alongside samples taken from the GMCP purification stages and purified SMCP (Fig. 3.4). The purified GMCP and SMCP samples demonstrated bands of proteolytic activity at 29kD (Fig. 3.4 lanes 3 and 5) which also coincided with the greatest activity in the isolated MMC extracts (Fig. 3.4 lane 4).

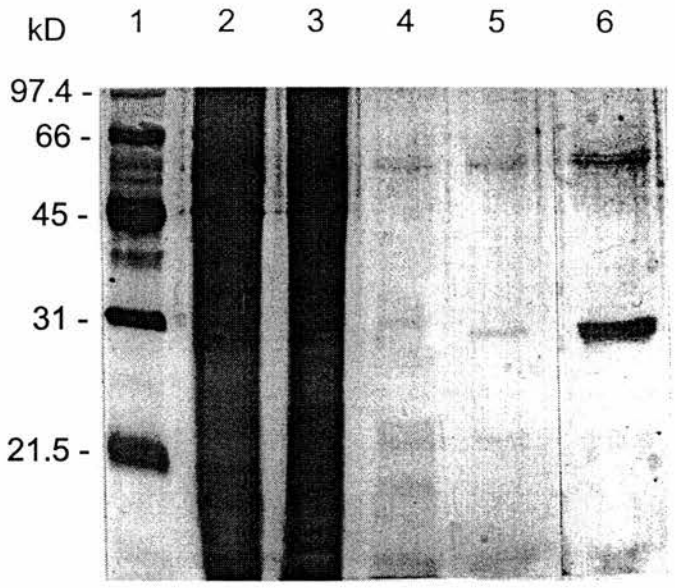


Fig.3.2a Silver stained SDS-PAGE gel of GMCP purification. Lane 1, reduced Biorad low molecular weight standards (relative molecular weights shown in kilodaltons (kD)); lane 2, jejunal tissue homogenised in 1.5M NaCl for SMCP ELISA analysis; lane 3, 1.0 M NaCl jejunal tissue extract; lane 4, CM-sepharose eluate; lane 5, MonoS purified GMCP (5µg/ml); lane 6, purified SMCP (34µg/ml).

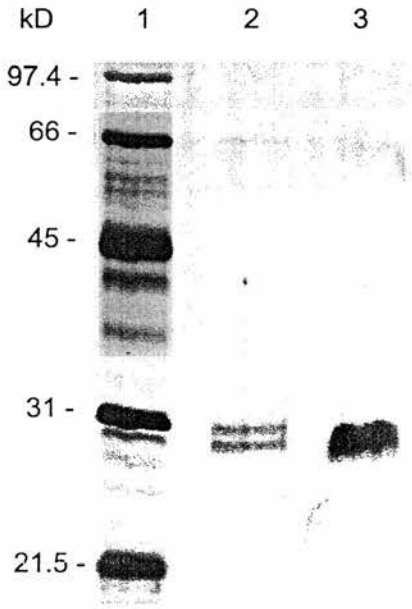


Fig. 3.2b Silver stained SDS-PAGE gel of immunoaffinity purified GMCP. Lane 1, reduced Biorad low molecular weight standards (relative molecular weights shown in kilodaltons (kD)); lane 2, purified SMCP (15 µg/ml) (note doublet) ; lane 3 immunoaffinity purified GMCP (15µg/ml)

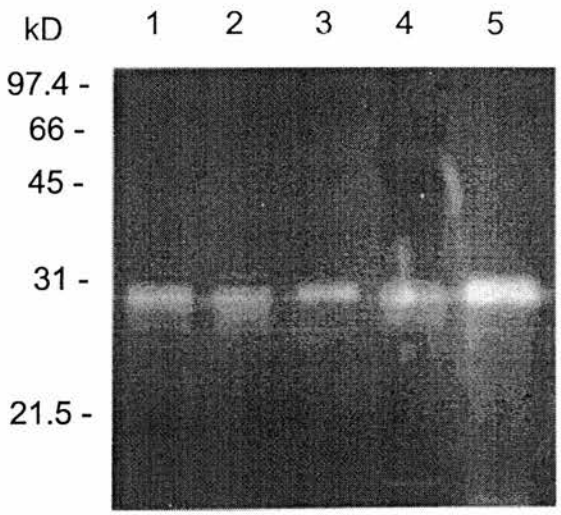


Fig. 3.3. Azocasein SDS-PAGE gel of GMCP purification, isolated MMC and *in vitro* derived BMMC. (relative molecular weights were defined by running non-reduced Biorad low molecular weight standards (not shown). Lane 1, 1.0M NaCl jejunal tissue extract; lane 2, CM-sepharose eluate; lane 3, MonoS purified GMCP (5 µg/ml); lane 4, 1.5M NaCl extracted MMC (1 x 10^6 cells); lane 5, purified SMCP (34µg/ml).

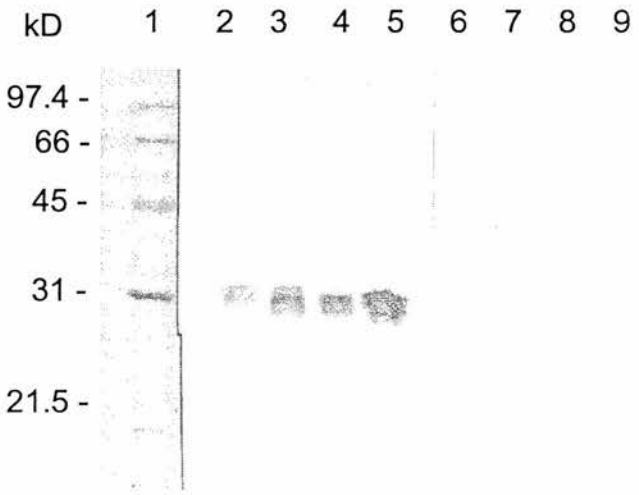


Fig. 3.4 Western Blot probed with rabbit polyclonal antibody raised against GMCP. Lane 1, Biorad reduced molecular weight standards; lane 2, mono S purified GMCP (7 µg/ml); lane 3, 1.5M NaCl extracted day 16 BMMC grown in rOvIL-3 and rOvSCF(1 x10^6 cells), lane 4, 1.5M NaCl extracted isolated goat MMC (1 x 10^6 cells); lane 5, purified SMCP (15µg/ml). Repeat samples run in lanes 6,7,8 & 9; these samples were probed with normal rabbit serum diluted 1: 500 as a negative control.

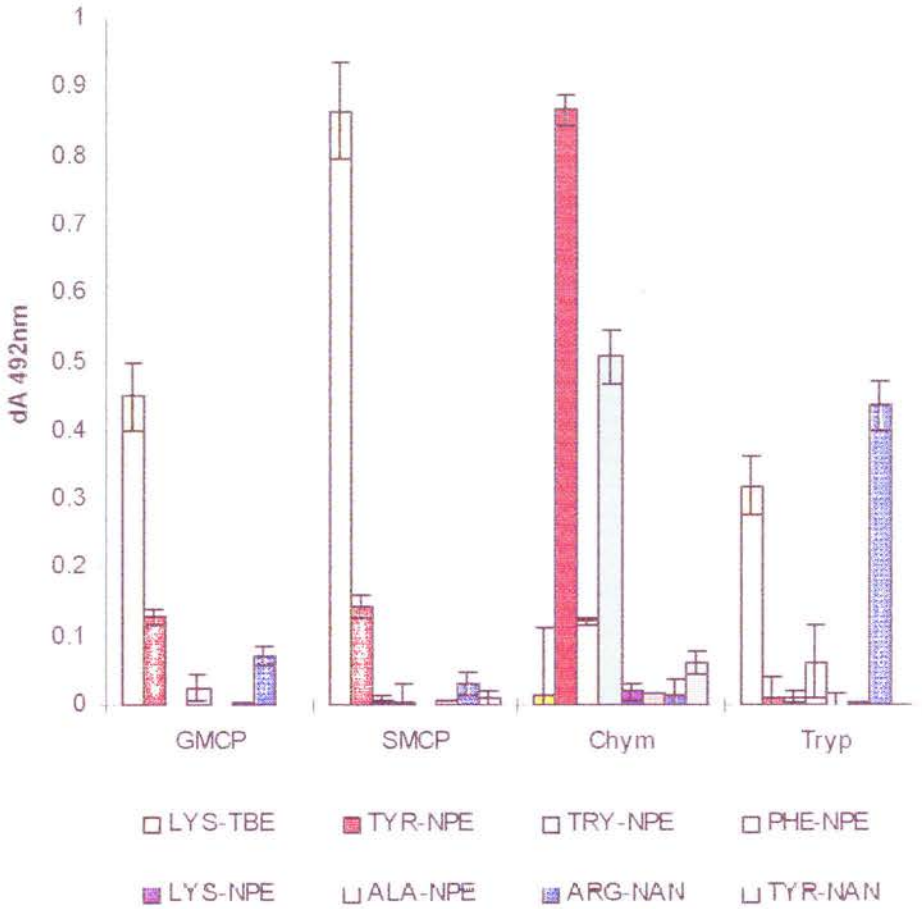


Fig.3.6 Comparative mean (±SD) substrate profile ODs for GMCP, SMCP, chymoptrypsin & trypsin activity. Results measured by dA at 492nm after 5 minutes incubation at room temperature for thiobenzyl ester and nitrophenol ester substrates or 2 hours incubation at 37 ^{O}C for nitroanilide substrates. Substrates tested: LYS-TBE (BLT) = carboxybenzoyl-L-Lysine thiobenzyl ester; TYR-NPE = carboxybenzoyl -L-Tyrosine nitrophenol ester; TRY-NPE = carboxybenzoyl-L-Tryptophan nitrophenol ester; PHE-NPE = carboxybenzoyl-L- Phenylalanine nitrophenol ester; ALA-NPE = carboxybenzoyl-L-Alanine nitrphenol ester; ARG-NAN = carboxybenzoyl-L-Argnine nitroanilide; TYR-NAN = carboxybenzoyl L-Tyrosine nitroanilide Results represent the mean valuse obtained for three experiments performed on duplicate samples.

Inhibitor	Stock Concentration	Final Concentration (mM)	GMCP	SMCP
			% Activity	
1,10 Phenanthrolline	1M	50	83	69
Pepstatin	100mM	5	82	79
E64 (thiol inhibitor)	1mM	0.05	100	100
Pms-F (serine inhibitor)	100mM	5	3	2
Serum (1:200)			1	0

Table 3.2 Mean % residual activity of GMCP and SMCP against BLT after 60 minutes incubation at room temperature with a selection of synthetic inhibitors and serum.

3.2.5 Comparative substrate & inhibitor profiles of GMCP & SMCP

Samples of purified GMCP were tested against synthetic substrates and inhibitor substances. These studies, run in parallel with equivalent samples of SMCP, bovine α-chymoptrypsin & trypsin (Fig.3.6 and Table 3.2) showed that GMCP has catalytic and inhibition properties that are almost identical to SMCP.

GMCP, SMCP and chymotrypsin all cleaved the chymotrypsin-associated substrate Cbz-L-Tyr NPE, whilst GMCP and SMCP also cleaved the trypsin-like substrate BLT which was also catalysed by trypsin but not chymotrypsin A (Fig.3.5). In addition, mean Km values calculated for both GMCP and SMCP in the presence of a range of concentrations of BLT (0.125-2 mM) gave the values 44 μM and 40.6 μM respectively which is in agreement with previous findings for SMCP (Pemberton *et al.*, 1997a). Inhibitor results (Table 3.2) showed that GMCP and SMCP were both completely inhibited by the serine esterase inhibitor PMS-F but were unaffected by the specific thiol proteinase inhibitor E-64. In addition, both enzymes were moderately inhibited (approximately 70-80%) by the chelating agent 1,10 phenanthrolline and by pepstatin but were again completely inactivated following incubation with goat serum or sheep serum respectively.

3.2.6 pH Optimum for GMCP activity

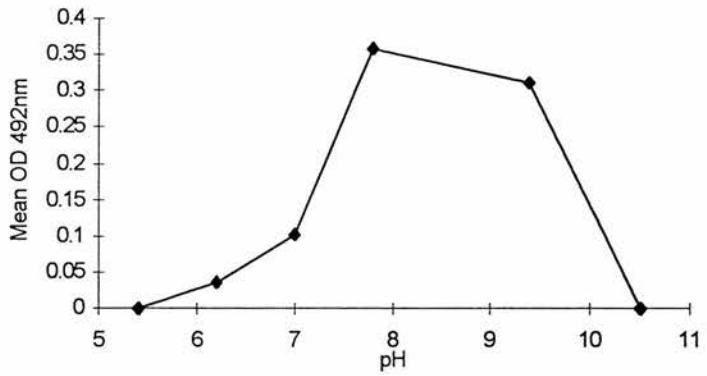


Fig. 3.7 Optimal pH titration for GMCP as measured by activity against BLT. Results shown are the mean values from samples tested in duplicate.

GMCP was found to have a broad pH optimum for activity against BLT with peak activity estimated to be around pH 7.8 (Fig. 3.7). This agrees with earlier results for SMCP against Cbz-L-Tyr NPE (Knox *et al.*, 1986).

3.2.7 Western Blot Analysis of GMCP & SMCP

Blotted samples of presumptive GMCP and SMCP probed with protein A purified polyclonal rabbit IgG anti-GMCP (adjusted to 1mg/ml and diluted to 1: 200 in PBS + 0.05% Tween 80 + 0.5 M NaCl) reacted with both GMCP and SMCP samples (Fig. 3.5 lanes 2 and 5) producing a thick single band for GMCP and a double band for SMCP at approximately 29kD. Extracts from day 16 goat bone marrow-derived mast cells (BMMC), cultured in rOvIL-3 and rOvSCF along with isolated goat MMC, also showed a single reactive band of similar weight (Fig. 3.5 lanes 3 and 4; see also Chapter 8) indicating that GMCP is present in both these cell types.

3.2.8 Amino acid sequence analysis of GMCP

GMCP samples purificd from 1.5M NaCl extracted homogenate samples on an anti-GMCP IgG column produced the highest yields of presumptive GMCP (>80% of the original tissue content). NH_2-terminal amino acid sequence analysis demonstrated a minimum 83% sequence homology with SMCP and bovine duodenase for the first 18 residues. The sample also showed a high degree of homology with other isolated rodent and murine mast cell proteases (Table 3.3).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
GMCP*	I	I	G	G	?	E	A	K	P	?	S	?	P	Y	M	A	F	L
SMCP*	I	I	G	G	H	E	A	K	P	H	S	R	P	Y	M	A	F	L
RMCPI	I	I	G	G	V	E	S	R	P	H	S	R	P	Y	M	A	H	L
RMCPII	I	I	G	G	V	E	S	I	P	H	S	R	P	Y	M	A	H	L
MMCPI	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L
MMCP2	I	I	G	G	V	E	A	K	P	H	S	R	P	Y	M	A	Y	L
MMCP3	I	I	G	G	V	E	S	R	P	H	S	R	P	Y	M	A	T	L
MMCP4	I	I	G	G	V	E	S	R	P	H	S	R	P	Y	M	A	H	L
MMCP5	I	I	G	G	T	E	C	I	P	H	S	R	P	Y	M	A	Y	L
MMCP6	I	V	G	G	H	E	A	S	E	S	K	W	P	W	Q	V	S	L
MMCP7	I	V	G	G	Q	E	A	H	G	N	K	W	P	W	Q	V	S	L
Bov Duodenase*	I	I	G	G	H	E	A	K	P	H	S	R	P	Y	M	A	F	L

Table 3.3 Comparative NH_2-terminal amino acid sequence data from isolated mast cell proteases in the goat, sheep, rat, mouse (sheep, rat and mouse data from Miller *et al.*, 1995) and bovine duodenase (data from Zamolodchikova *et al.*, 1995b). Amino acids shown in standard single letter notation. ? = undetermined residue * GMCP, SMCP and bovine duodenase share a minimum 83% homology over the first 18 residues.

3.2.9 *Immunohistochemical analysis of MMC and GL in tissues*

Table 3.4a show counts obtained from serial sections of abomasal tissues from the ADCM challenged adult goats (does and bucks), worm naive kids, ADCM challenged lambs and, single primary challenged lambs. Table 3.4b shows counts obtained from serial sections of jejunal tissues from the ADCM challenged adult goats and worm naive kids. The cells were stained with 0.5% toluidine blue and carbol chromotrope for MMC and GL respectively as well as polyclonal rabbit anti-GMCP and polyclonal rabbit anti-SMCP antibody. Cells staining positive with the anti-GMCP antibody shared the same

anatomical distribution, within the lamina propria of the mucosa, as the toluidine blue stained cells, whilst morphologically they also exhibited a focal pattern of staining consistent with the presence of cytoplasmic granules (Figs. 3.1c and d; 3.8a and b). In addition, a number of intra-epithelial cells also stained positively (Fig. 3.8a and b) although more faintly than the cells within the submucosa. These fainter staining cells exhibited similar morphology and tissue location as the carbol chromotrope stained GL (Fig. 3.1e)

Group (n)	Toluidine blue (MMC)	Carbol chromotrope (GL)	Total MMC/GL	Anti-GMCP	Anti-SMCP
Does (6)	10.0 (±4.7)	12.6 (±10.1)	22.6(±12.6)	7.3 (±3.7)	8.9 (±3.7)
Bucks (6)	7.2 (±1.7)	9.1 (±4.0)	16.3 (±4.9)	5.2 (±3.0)	5.5 (±2.5)
Kids (3)	1.8 (±0.6)^{a}	0.3 (±0.3)^{a}	2.1 (±1.3)^{a}	1.1 (±1.0)^{a}	1.3 (±2.3)^{a}
Lambs (6)	19.6 (±4.5)^{b}	5 (±4.0)	24.6 (±6.2)	18.3 (±4.7)^{b}	18.8 (±3.3)^{b}
Primary challenged Lambs*(3)	2.5 (±1.3)^{a}	1.8 (±1.7)	4.3 (±1.6)^{a}	3.3 (±0.9)^{a}	3.9 (±1.1)^{a}

Table 3.4a Mean (± SD) cell counts for abomasal tissues from goats and lambs. Represented as numbers of cells per 0.2mm^2 Total MMC/GL represents the sum of the results obtained for the toluidine blue (MMC) and carbol chromotrope (GL) stained sections. * primary challenged lambs who received a single 50,000 *T. circumcincta* challenge ten days prior to slaughter. Superscripts show values that are significantly different ($p < 0.05$). a = values significantly less than corresponding ADCM challenged animals of the same species; b = values significantly higher than those obtained for the does, bucks, kids or naive lambs.

Group (n)	Toluidine blue (MMC)	Carbol chromotrope (GL)	Total MMC/GL	Anti-GMCP	Anti-SMCP
Does (6)	13.8 (±2.0)	9.4 (±5.5)	23.2 (±7.2)	15.6 (±2.0)	14.9 (±1.6)
Bucks (6)	16 (±0.9)	8.7 (±2.0)	24.7 (±4.9)	15.8 (±3.5)	17.2 (±1.6)
Kids (3)	14.6 (±2.4)	0.6 (±0.5)^{a}	15.3 (±2.4)^{a}	7.6 (±2.9)^{a}	6.3 (±3.4)^{a}

Table 3.4b Mean (±SD) cell counts for jejunal tissues from goats only represented as numbers of cells per VCU. Total MMC/GL represents the sum of the results obtained for the toluidine blue (MMC) and carbol chromotrope (GL) stained sections. Superscripts show values that are significantly different ($p < 0.05$). a = values significantly less than corresponding ADCM challenged does and bucks.

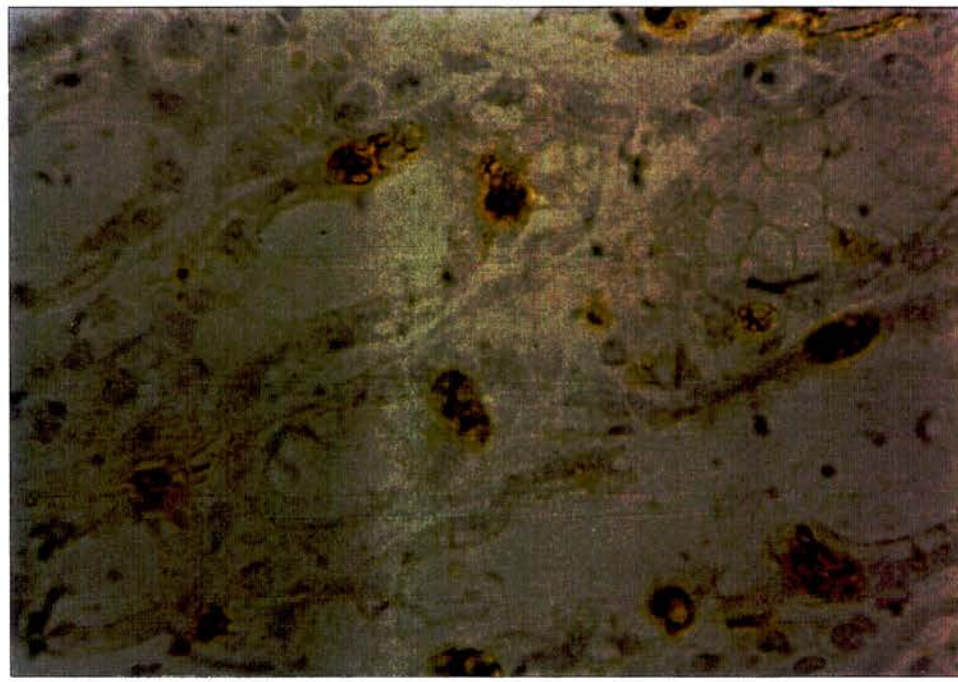


Fig 3.8a Goat abomasal tissue incubated with polyclonal anti-GMCP. Note the granular cytoplasmic morphology of the MMC and the fainter staining GL.4% Paraformaldehyde fixed tissue (x 400).

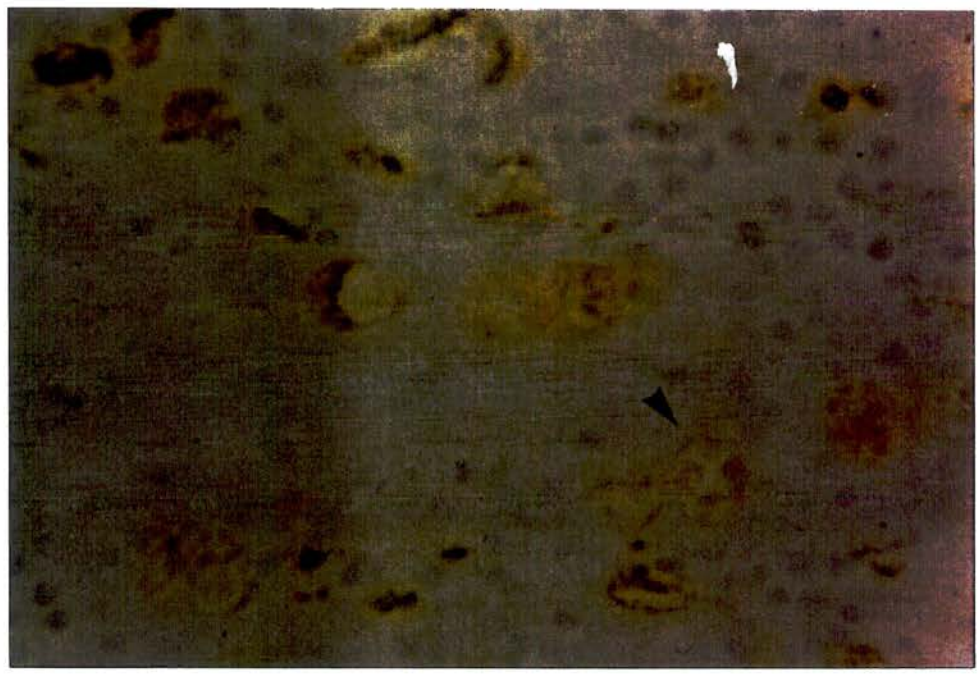


Fig 3.8b Goat jejunal villus crypt incubated with polyclonal anti-GMCP. Note the fainter staining intra-epithelial cells (arrow), compare with the GL shown in Fig. 3.1e. 4% Paraformaldehyde fixed tissue (x 400).

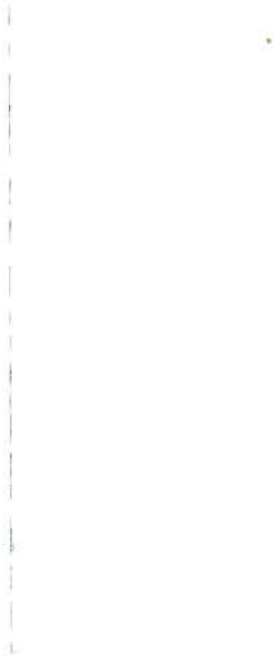


Fig 3.8c Goat abomasal tissue incubated with normal rabbit IgG. 4% Paraformaldehyde fixed tissue (x 100).

Fig 3.8d Goat jejunal villi incubated with normal rabbit IgG. 4% Paraformaldehyde fixed tissue (x 100).

Abomasal cell counts (Table 3.4a) demonstrated that, with the exception of carbol chromotrope stained cells in the lambs, ADCM challenged animals from both species showed significant increases in the numbers of cells stained by all methods when compared to worm naive kids or primary challenged lambs. The ADCM challenged lambs also contained significantly higher numbers of toluidine blue, anti-GMCP and anti-SMCP staining cells than the ADCM challenged goats ($p < 0.05$). However, both the does and bucks contained higher mean numbers of carbol chromotrope staining cells, which meant that there were no significant differences in terms of the 'total mast cell count' (ie the combined toluidine blue (MMC) and carbol chromotrope (GL) stained cell counts) between the ADCM challenged lambs and goats. In the jejunum (Table 3.4b), there were significantly greater numbers of carbol chromotrope, anti GMCP and anti-SMCP positive cells in the does and bucks compared to the kids, although there were no significant differences observed in the toluidine blue cell counts. Regression analyses comparing abomasal toluidine blue cell counts with abomasal GMCP-positive cell counts from all animals demonstrated a high degree of correlation ($R^2 = 0.95$, $p < 0.0001$) whilst a less strong, but still significant relationship, was also demonstrated between abomasal total cell counts (MMC/GL) and anti-GMCP cell counts ($R^2 = 0.75$, $p < 0.0001$). In addition, the relationships between the numbers of anti-GMCP positive and anti-SMCP positive cells in the abomasal tissues from both species and the goat jejunal tissues were also highly correlated ($R^2 = 0.98$, $p < 0.0001$ and $R^2 = 0.81$, $p < 0.001$ respectively).

3.2.10 Production of monoclonal antibodies to GMCP

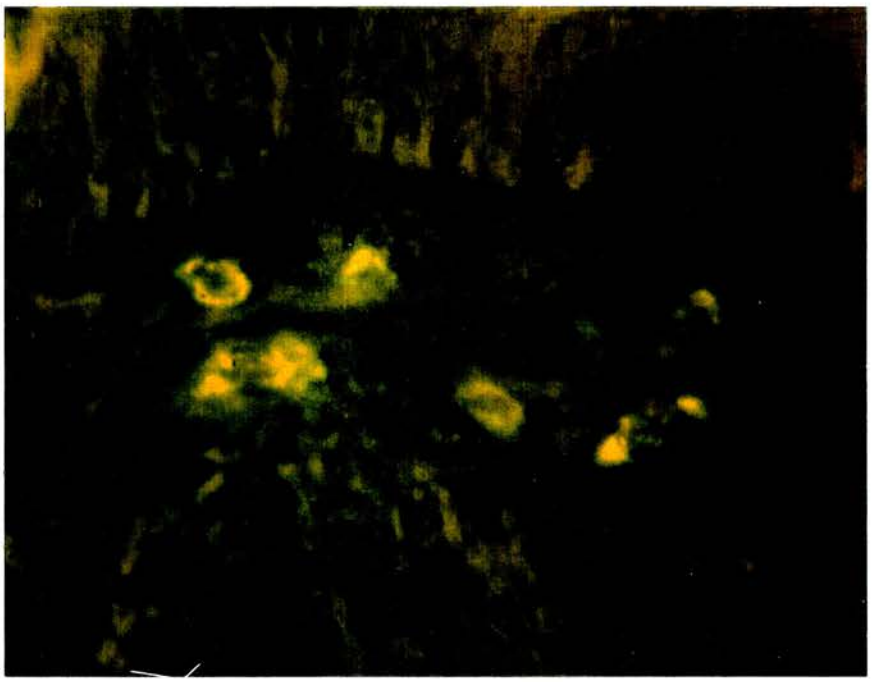
The hybridoma cells originally produced 5 clones whose supernatants stained cells on tissue sections that corresponded in distribution and morphology to cells stained with toluidine blue or incubated with polyclonal anti-GMCP. The parent hybridoma colonies

were allowed to grow to confluency, before being harvested and stored in liquid nitrogen. Cells from two of the positive colonies (3E3 and 5C1) were subcloned twice and the resulting supernatants from single cell-derived clones retested on tissue sections. On the basis of the intensity, morphology and anatomical location of cells stained, one subclone (3E3/E3/E4), was cultured to produce sufficient cells for ascites production as outlined in 2.4.2. Harvested ascites fluid was tested on tissue sections before purifying on a protein G column and biotinylating as outlined in 2.4.2

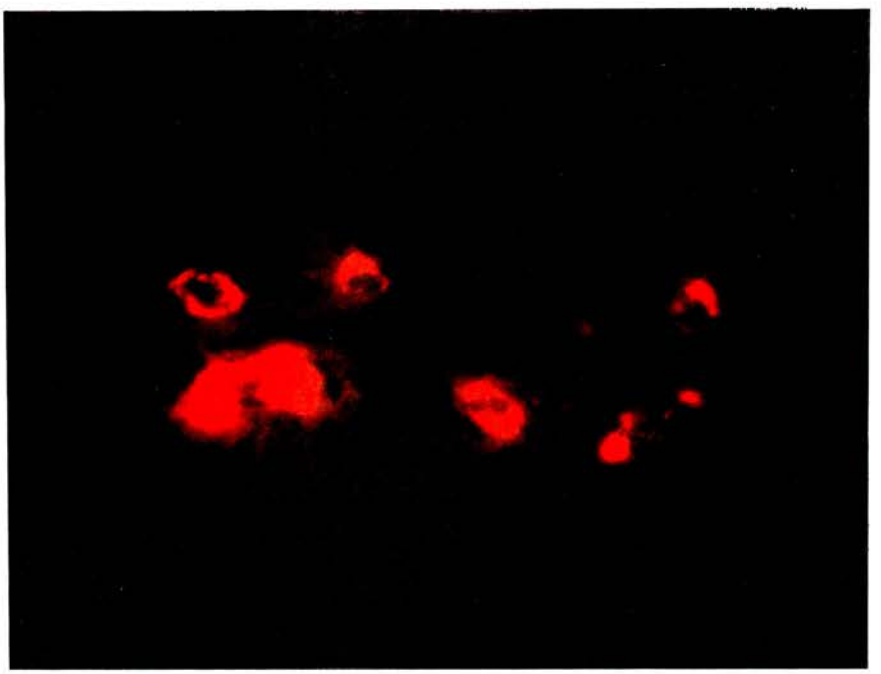
3.2.11 Dual immunofluorescence using monoclonal anti-GMCP and polyclonal anti-SMCP

Figures 3.9a and b show FITC positive cells stained with monoclonal anti-GMCP and TRITC positive cells stained with polyclonal anti-SMCP respectively. This demonstrates the cross reactivity shown by the two antibodies as they stain the same MMC populations within the lamina propria. This same pattern of staining was also seen in the abomasum. Control slides, where monoclonal anti-GMCP and polyclonal anti-SMCP were replaced with a non-related murine monoclonal antibody to border disease virus (VPM-12) and normal rabbit serum respectively were negative (Figs. 3.9 c and d). Unfortunately, GL auto-fluoresced on the negative control slides despite attempts to block this by preparing the tissues in modified Bouin's fixative and pre-treating the slides with DAB (Figs. 3.9 c and d). It was therefore impossible to determine, by dual fluorescence, whether they stained positively with both antibodies as well..

a



b



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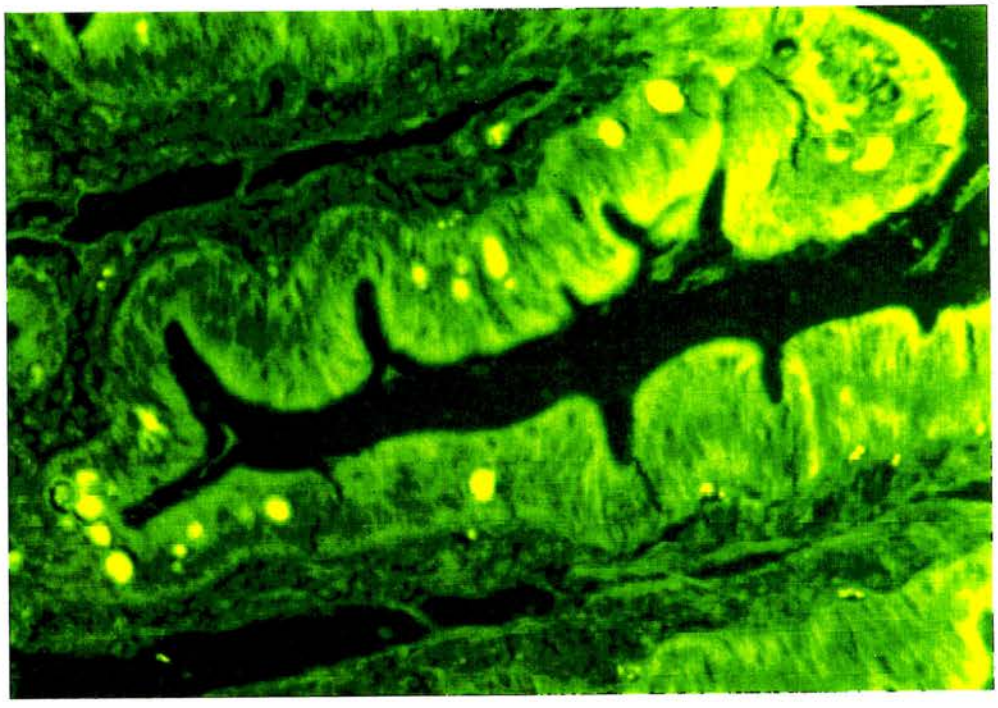

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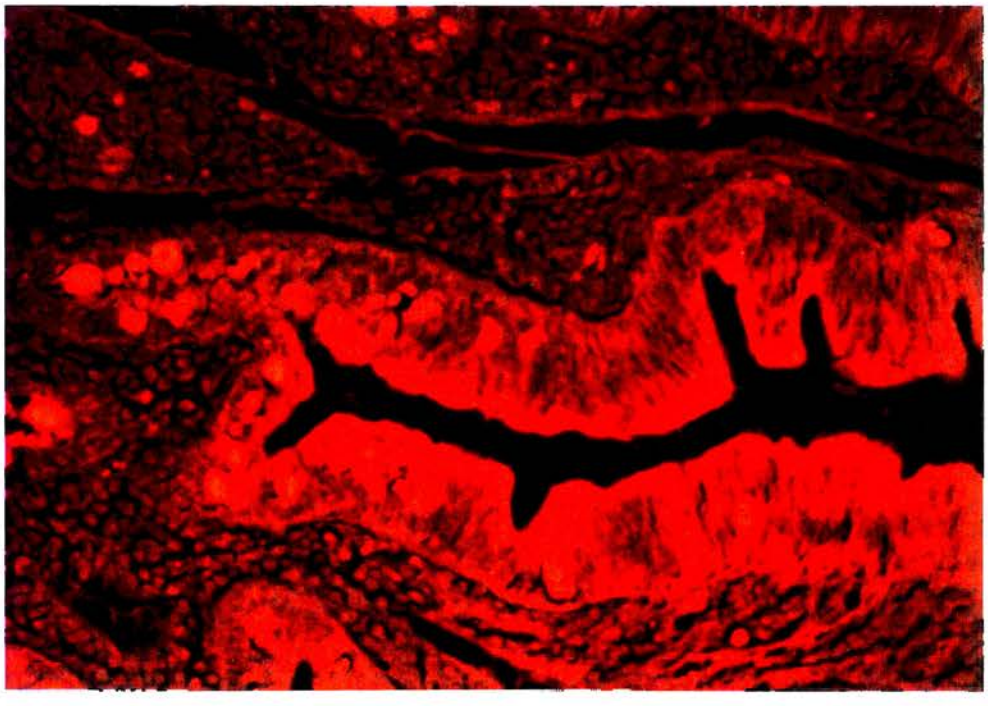


Fig. 3.8 Dual immunofluorescence staining of MMC in goat jejunal tissues. a) Cells staining positive in a jejunal villus tip with mouse monoclonal anti-GMCP and FITC conjugated anti-mouse IgG. b) Same cells staining positive with rabbit polyclonal anti-SMCP and TRITC conjugated anti-rabbit IgG. c) and d) jejunal tissues incubated with VPM-12 (c) and normal rabbit IgG (d) respectively, Note the lack of MMC staining and the auto-fluorescence of intra-epithelial GL. Modified Bouin fixed tissue (a and b x 400; c and d x 250)

3.2.12 GMCP ELISA

Fig 3.10 shows the results of an experiment to determine the optimal concentration for polyclonal capture antibody to GMCP. Duplicate rows on ELISA plates were coated with polyclonal IgG anti-GMCP at 16 µg/ml, 8µg/ml, 4µg/ml and 2 µg/ml in CO_3 buffer as outlined in 2.12.1. Each set of coated wells was then incubated with GMCP samples diluted in PBS + 0.5M NaCl to provide a concentration range from 5ng/ml to 70 ng/ml. GMCP binding to the capture antibody was detected using biotinylated monoclonal anti-GMCP as described in 2.12.1. The results demonstrate a significant reduction in O.D., indicating a loss in sensitivity, when polyclonal IgG anti-GMCP is used at less than 8µg/ml. However, the sensitivity was not significantly enhanced by coating at 16µg/ml, therefore, plates were routinely coated with polyclonal antibody at 10µg/ml. Fig. 3.11 shows the mean OD results from a standard curve titration for purified GMCP on plates that had been coated with polyclonal anti-GMCP at 10µg/ml. The most linear part of the curve was found to lie between approximately 1ng/ml and 15 ng/ml. A standard curve range of 0.5 to 12 ng/ml similar to that described previously by Huntley *et al.* (1987) for an SMCP ELISA, was employed in subsequent assays. Fig. 3.12 demonstrates the cross reactivity shown by the anti-GMCP antibodies used in the GMCP ELISA, when testing samples containing known stock concentrations of purified GMCP or SMCP. Samples were diluted in PBS + 0.02% Tween20 to produce a 0.5-12 ng/ml range of concentrations. No significant differences ($p > 0.05$) were observed in the mean OD 492_{nm} results obtained from either species at each concentration, demonstrating total antigenic cross reactivity between SMCP and GMCP in this ELISA.

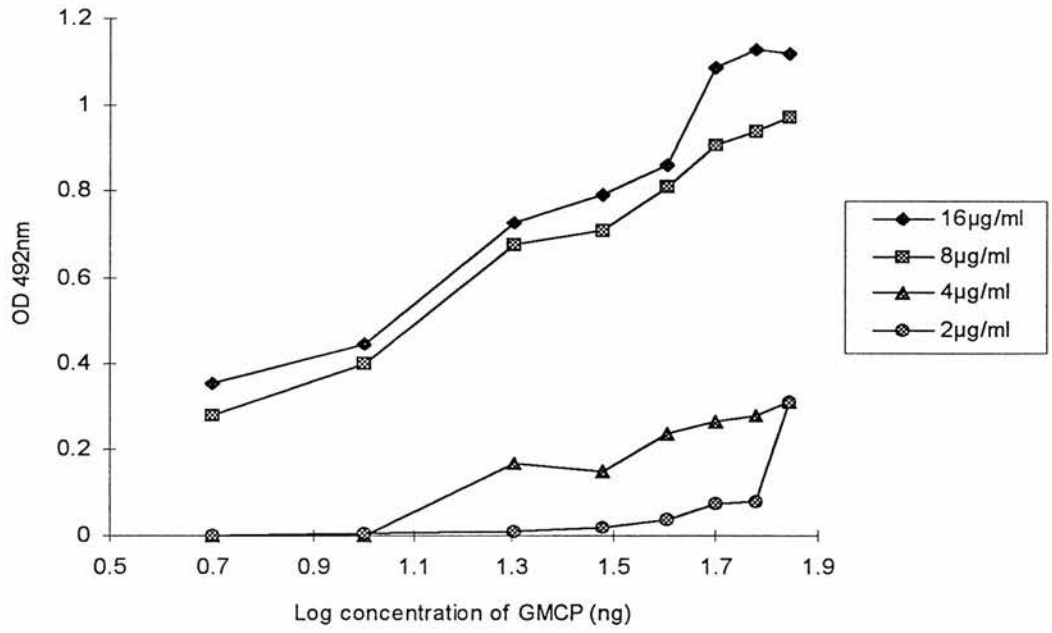


Fig. 3.10 The effects of concentration of coating polyclonal rabbit anti-GMCP on the sensitivity of a double antibody sandwich ELISA for GMCP. Biotinylated monoclonal anti-GMCP antibody was used at 1:200 dilution of stock. The colour reaction was allowed to develop for 30 minutes before terminating with 2.5M H_2SO_4 and measuring the blank corrected OD 492nm. Each point represents the mean of duplicate tests.

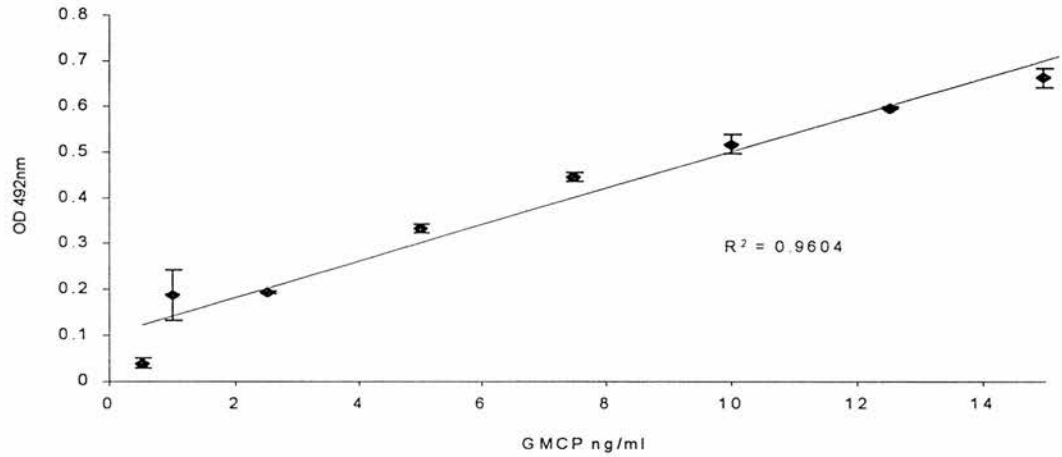


Fig 3.11 Standard curve titration for purified GMCP in the GMCP ELISA. The graph shows a range of dilutions of GMCP from 0.5ng/ml to 15ng/ml. The most linear part of the curve used for measuring GMCP concentrations lies between 0.5ng/ml and 12 ng/ml (as determined by Dynatech data analysis software). Each data point represents the mean (± SD) of 4 tests.

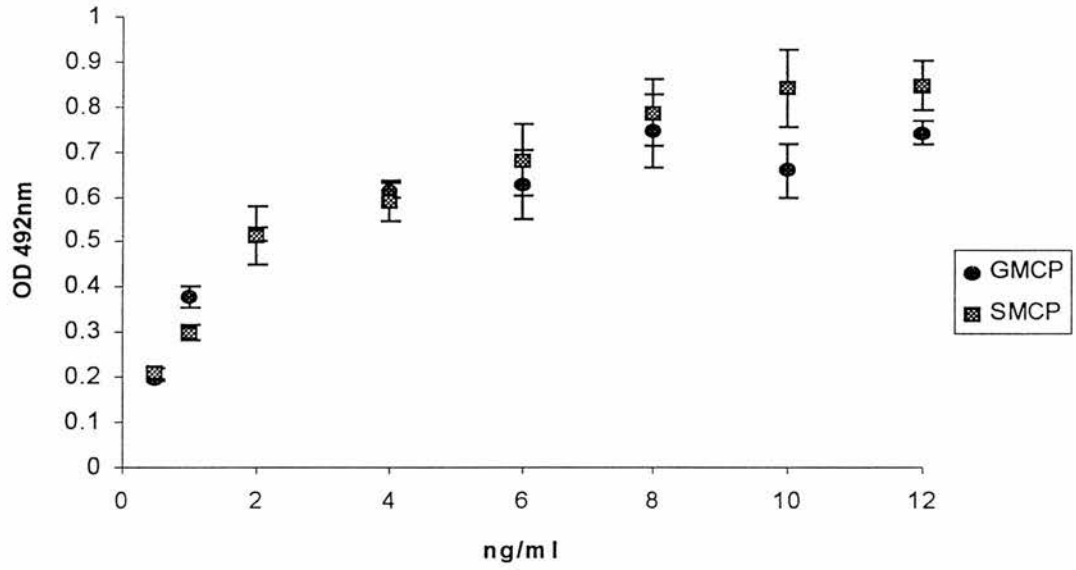


Fig. 3.12 Comparative OD 492nm results obtained for a 0.5 - 12ng/ml dilution range of stock GMCP and SMCP samples. No significant differences were observed in the absorbances measured for each sample. Each data point represents the mean (± SD) of 4 tests

3.4 Discussion

This chapter describes, for the first time, the isolation and purification to homogeneity of a goat mast cell protease (GMCP). The enzyme was purified from samples of goat jejunum that contained the highest levels of putative GMCP as determined by an ELISA for SMCP. Following preliminary investigations to determine the concentration of NaCl required to achieve maximal tissue protease extraction, GMCP was extracted from homogenised tissues by solubilising it in a high salt (1.0M NaCl) buffer. This possibly acts to release GMCP from the granule glycosaminoglycan matrix to which it is ionically bound when the mast cell is in a quiescent state (Schwartz and Austen, 1984). The requirement for NaCl in the extraction buffer was in common with other relatively insoluble mast cell proteases such as SMCP and RMCP I, both of which

which need high salt extraction buffers for purification (Huntley *et al.*, 1986; Yurt & Austen, 1977). Subsequent cation exchange chromatography steps further categorised the enzyme as a basic protein, whilst SDS-PAGE analysis showed that it had a molecular weight of approximately 29kD, which was in common with SMCP. Pierce protein quantification and SMCP ELISA analysis of each purification step indicated that only low μg level quantities of protease were present in the goat tissues and FPLC column eluates. These findings agree with earlier results for GMCP quantification in tissues as previously determined using the ELISA for SMCP (Huntley *et al.*,1995).

The mast cell origin of GMCP can be deduced on the basis of five separate findings. Firstly, tissue staining with the substrate used to monitor the purification of GMCP (BLT) was localised to cells also staining positive with toluidine blue, which is specific for mast cell protoglycans (Enerback, 1966a). Secondly, azocasein substrate gels loaded with samples of purified GMCP produced a band of proteolytic activity at 29 kD, which was also seen in samples of cell extracts from density gradient purified goat jejunal cells that were enriched for MMC and GL (see Chapter 8). Thirdly, Western blot analysis of purified GMCP samples, isolated MMC/GL extracts and *in vitro* derived bone marrow mast cell (BMMC) extracts all exhibited a protein bands at 29-30kD when probed with polyclonal rabbit IgG anti-GMCP. In addition, samples of purified SMCP also stained with this antibody indicating the presence of cross reactivity between the two proteases. Fourthly, cell counts for goat and sheep tissue sections stained with polyclonal rabbit IgG anti-GMCP correlated with counts from the same sections stained with toluidine blue for MMC, whilst both populations of stained cells were found to be localised to the lamina propria and exhibited similar staining morphology. Finally, a high degree of homology between NH_2-terminal amino acid sequences obtained for immunoaffinity purified GMCP and SMCP (Pemberton *et al.*, 1997a) provided, together with the above evidence, confirmation that GMCP represents a caprine mast cell-specific protease.

The presence of BLT and polyclonal anti-GMCP staining cells which were localised in the epithelium of the jejunum as well as the abomasum provided evidence that goat GL may be derived from MMC. These cells also demonstrated a similar morphology and tissue distribution to GL stained with carbol chromotrope. The fact that the cells were particularly faint staining may have resulted from them having already degranulated and released the majority of their protease content. This in turn, may have accounted for poorer correlations obtained when total MMC/GL cell counts from toluidine blue and carbol chromotrope stained abomasal tissues were compared with the results from equivalent tissues stained with anti-GMCP. An alternative rationale is that caprine GL represent a heterogeneous population in which protease is absent in some cells, or that they contain protease which is antigenically distinct from GMCP.

Three further significant results were obtained from the histochemical and immunohistochemical analyses of the sheep and goat tissues. Firstly, the numbers of mast cells increased significantly in all animals exposed to the ADCM challenge indicating that intestinal mastocytosis occurs in both species following nematode challenge. Secondly, whilst the ADCM challenged lambs generated significantly more MMC than the goats, the numbers of total mast cells (MMC and GL) were not significantly different due to proportionately greater increases in GL numbers in the ADCM challenged goats. Earlier findings (Huntley *et al.*,1995), demonstrated greater numbers of GL in the gastro-intestinal tissues of goats when comparing ADCM challenged does and ewes, whilst significantly higher total worm burdens were also recovered from the does at post mortem. Parasite numbers were not recorded in the current experiment, however these previous findings, along with the evidence that at least some caprine GLs are related to MMC suggests that there may be greater or more prolonged antigenic stimulus for increased GL formation from MMC in goats. Finally, there was also an extremely high correlation between the cells of similar morphology and tissue location staining positive with anti-GMCP and anti-SMCP in both species. This

indicates that a high degree of cross reactivity occurs between the antibodies to both proteases suggesting that both enzymes are closely related in terms of immunogenicity, structure and, possibly, biochemical function.

Results from biochemical characterisation studies including semi-quantitative substrate and inhibitor assays demonstrated that both GMCP as well as SMCP, as their 'chymase' nomenclature would suggest (Miller *et al.*, 1995), possess catalytic activity against some chymotrypsin-associated substrates. This included Cbz-L-Tyr NPE which contains an aromatic P1 residues at its catalytic site (see general introduction). However, somewhat unusually, GMCP and SMCP also demonstrated a partial 'trypsin-like' reactivity restricted towards BLT, which contains a lysine P1 residues at its catalytic site. Indeed, the catalytic activity against this particular substrate was so rapid that it was subsequently chosen for routinely monitoring GMCP activity during FPLC purification. GMCP, like SMCP, was also relatively unaffected by the specific thiol-proteinase inhibitor E-64 whereas it is fully susceptible to PMS-F indicating that both enzymes are serine esterases. Moreover, both GMCP and SMCP were completely inhibited after incubating in normal goat or sheep serum indicating that they are both susceptible to serum derived protease inhibitors (serpins) present *in vivo*. These may include alpha-1-proteinase (alpha-1-antichymotrypsin) and alpha-2-macroglobulin inhibitors which have been demonstrated in sheep serum (A. Pemberton personal communication, Pemberton *et al.*, 1997b) and/or the recently characterised goat serine protease inhibitors, elastasin and contrapsin which have been shown to be similar to human alpha-1-antichymotrypsin (Potempa, Enghild and Travis, 1995). In previous studies, both SMCP and bovine duodenase have also been shown to be susceptible to soyabean trypsin inhibitor (Pemberton *et al.* 1997b, Zamolodchikova *et al.*, 1995a) further emphasising their 'trypsin-like' biochemical properties. Catalytic activity against BLT was also noted in samples of purified MMC/GL that had been lysed in a 1.5M NaCl buffer at pH 7.5. This, along with the results from the BLT staining of tissues discussed

above, implies that GMCP is stored in an active form within the cell, whilst the granular pattern of staining present in tissue MMCs probed with antibody to GMCP (Fig. 3.7 a and b) appears to localise the enzyme to the cytoplasmic granules. Overall, these results indicate that GMCP, in common with SMCP is present in MMC where it is found as a biochemically active, granule-associated, serine protease exhibiting both chymotrypsin-like and partial trypsin-like substrate specificity. This property of dual substrate specificity is a relatively novel finding, associated with certain human and ruminant serine proteases and has recently been ascribed to human cathepsin G (Hof *et al.*, 1996), SMCP (Pemberton *et al.*, 1997a) and bovine duodenase (Zamolodchikova *et al.*, 1995a).

NH_2-terminal amino acid sequence results for the first 18 residues of an immunoaffinity purified sample of GMCP demonstrated a minimum 83% homology with SMCP and bovine duodenase and 33%-66% homology with other previously sequenced rodent and murine mast cell proteases. Work is currently underway to determine the complete amino acid sequence for this protease through cDNA sequence analysis obtained from *in vitro* derived BMMC mRNA (see Chapter 5). When complete, it is likely that the well conserved serine esterase-associated catalytic triad of His-57, Asp-102 and Ser 195 common to chymotrypsin A and other completely sequenced mucosal mast cell chymases such as MMCP 1 & MMCP 2 (Huang, Blom and Hellman 1993; Serafin, Reynolds, Rogelj, Lane, Conder, Johnson, Austen and Stevens, 1990) and RMCP I (Le trong, Parmelee, Walsh, Neurath and Woodbury, 1987) will also be found. Sequence analyses may also provide more information on the presence of potential glycosylation sites which may account for doublet bands seen in some preparations of SMCP and immunoaffinity-purified GMCP isolated from tissues (Fig. 3.2a and b), as well as the presence of certain charged amino acid residues within the active site which could account for the presence of dual specificity (Hof *et al.*, 1996).

Attempts at constructing an ELISA with polyclonal and monoclonal antibodies to GMCP were also successful, with optimal concentrations of polyclonal capture antibody

enabling the assay to detect as little as 1ng/ml of GMCP. Given the high degree of cross-reactivity shown by polyclonal and monoclonal anti-GMCP and polyclonal anti-SMCP antibodies (Figs. 3.5 and 3.9), it is perhaps not surprising to find that there were no significant differences in the ability of the GMCP ELISA to detect either GMCP or SMCP. However, this work validates previous results obtained using SMCP antibodies for the detection of GMCP in goat tissues (Huntley *et al.*, 1995).

Having isolated and characterized GMCP, further studies were performed to analyse its distribution in mast cell populations, its molecular characteristics through cDNA sequencing and its production in response to host infection with nematode parasites.

CHAPTER 4

THE DISTRIBUTION OF GOAT MAST CELLS, GLOBULE LEUKOCYTES AND GOAT MAST CELL PROTEASE.

4.1 Introduction

Currently, very little is known about the distribution of mast cells or globule leukocytes in goat tissues. Histochemical studies carried out by Rahko (1972a and b) demonstrated the presence of mast cells and GL in the bile ducts of goats during natural infections of fascioliasis, whilst Huntley *et al.* (1995) also demonstrated their presence in the abomasa and jejuna of does infected with *T. circumcincta* and *T. vitrinus*. However, histochemical studies on the distribution of these cells throughout the rest of the gastro-intestinal tract as well as in other tissues have yet to be carried out. Moreover, as outlined in the general introduction, mast cells from a number of species including rodents, mice, humans and most recently sheep, have been shown to possess phenotypic heterogeneity in terms of their tissue location, histochemical, biochemical and functional properties (reviewed Galli, 1990 and Huntley, 1992).

In this respect, studies investigating the expression of antigenically distinct neutral granule proteases in mast cell populations has led to the identification of MMC and CTMC subsets in the rat (Gibson and Miller, 1986; Gibson *et al.*, 1987; Huntley, Mackellar, Newlands, Irvine and Miller, 1990), mouse (Miller, Huntley, Newlands, Mackellar, Irvine, Haig, Macdonald, Lammas, Wakelin and Woodbury, 1989) and man (Schwartz, 1989). In the rat, RMCP II predominates in intestinal MMC, whilst RMCP I is primarily located in CTMC found in sites other than mucosal tissues. In mice, MMCP 1 is only expressed in MMC, whilst MMCP 4 is principally found in CTMC (Reynolds *et al.*, 1990). In humans, there are at least two mast cells subsets, one that contains a tryptase and a chymase (MC^{TC}), and one that contains only tryptase (MC^{T}). Most tissues appear to contain a mixture of both phenotypes, although MC^{TC} cells are predominantly found in the skin and small intestinal submucosa whereas MC^{T}s are chiefly found in the lung and small intestinal mucosa (Schwartz, 1989). Recent work by Sture *et al.* (1995) demonstrated heterogeneity amongst sheep mast cells on the basis of their SMCP content. Two populations of

sheep mast cells were defined, those in gastrointestinal and other tissues where the majority of mast cells stained positive for SMCP (equivalent to an MMC subset) and those present in the skin (a putative CTMC subset) that was deficient in SMCP.

The work presented in Chapter 3 demonstrated the isolation, purification and characterization of a mast cell-specific protease from goat tissues (GMCP) that is analogous to SMCP. The development of polyclonal antibodies to GMCP now provides an opportunity to examine the distribution of mast cells and GL within goat tissues. This will be achieved using both histochemical techniques to stain mast cells and GL as well as immunohistochemistry which may provide evidence for phenotypic heterogeneity in terms of the distribution of GMCP. In addition, the development of an ELISA for GMCP will allow assessments to be made on the concentration of GMCP in various tissues. Finally, the kind gift of a polyclonal rabbit antibody to human mast cell tryptase (from Professor K. F. Austen via Professor H. R. P. Miller), which has been shown to cross react with a putative tryptase enzyme in sheep mast cells (H. R. P. Miller. personal communication), will also allow preliminary investigations into the presence or absence of a putative goat mast cell tryptase. The distribution of tryptase positive cells will be examined in relation to the distribution of GMCP-positive cells to determine whether a situation analogous to MC^T and MC^{TC} cell heterogeneity described in humans, also occurs in the goat.

4.2 Materials and methods

4.2.1 Animals

Three adult (three to four year old) female Scottish cashmere goats were used for the study. They had undergone exposure to nematode infections at pasture before being housed approximately six months prior to the start of the experiment. In order to stimulate an intestinal mast cell response, they were dosed with 2000 *T. circumcincta* L_3 and 2000 *T. vitrinus* L_3 weekly *per os* as described in Chapter 2.9.1.

4.2.2 Processing of tissues

The animals were killed as described in Chapter 2.3.3 before identifying and harvesting the following tissues from similar locations in each animal: abomasum (abomasal fold), duodenum, jejunum, ileum, colon, mesenteric lymph node, trachea, lung (right caudal lobe), bronchial lymph node, spleen, liver (ventral margin of the left lobe), skin (glabrous skin from ventral abdomen) and heart (left ventricle). Harvested tissues were fixed in 4% paraformaldehyde/PBS or weighed, homogenised and stored at -20 ^{o}C for ELISA analysis as described in Chapter 2.8.1 and 2.3.3 respectively. 4 µm serial sections were prepared for histochemical and immunohistochemical staining as described in Chapter 2.8.1

4.2.3 Staining and enumeration of cells in tissue sections.

Sequential sections were cut and stained with toluidine blue pH 0.5 (Enerback, 1966; see 2.8.3) for mast cells (MC), carbol chromotrope for GL (Lendrum, 1944; see Chapter 2.8.4), polyclonal rabbit IgG containing antibody to GMCP or affinity purified polyclonal rabbit antibody to human tryptase (Chapters 3 & 2.8.7). Negative controls for the immuno-localisation incorporated normal rabbit serum diluted 1:500 in place of the primary antibody. Cell counts were carried out on paired segments of tissue from related areas on sequential sections as described in Chapter 2.8.9. Counts

from intestinal tissues distal to the abomasum were quoted as numbers of positive cells per VCU whilst counts from all other tissues were quoted as numbers of positive cells per $0.2mm^2$.

4.2.4 ELISA for GMCP

Tissue homogenates prepared in a 1.5M NaCl 20mM Tris buffer as described in Chapter 2.3.3 were tested on a double antibody sandwich ELISA for GMCP as described in Chapter 3. using the protocol outlined in Chapter 2.12.1. Final results were quoted as μg GMCP/g wet weight tissue.

4.3 Results

The mean (±SD) results for the toluidine blue, carbol chromotrope, anti-GMCP and anti- human tryptase cell counts along with their respective tissue GMCP concentrations are shown in Table 4.1. Overall, toluidine blue, anti-GMCP and anti-human tryptase staining cells as well as detecTable levels of GMCP were identified in every tissue tested except the trachea. In this tissue, mast cells were not found on any of the sections examined. Histochemical staining with carbol chromotrope identified GL in gastro-intestinal tissues only, where they were found in all areas except the colon. As previously (Chapter 3), both intestinal mast cells and GL stained positive using antibody to GMCP (as well as antibody to human tryptase, Fig. 4.3). Therefore, for comparative purposes the results for toluidine blue stained MMC and carbol chromotrope stained GL in gastro-intestinal tissues were combined to give a 'total mast cell' count. In the gastro-intestinal tissues the highest total mast cell counts were obtained for the jejunal tissues (26.2 cells/VCU) which also corresponded with the highest counts for tissues stained with anti-GMCP (18.9 ± 0.3 cells/VCU). Apart from the intestine, the highest mast cell counts obtained for toluidine blue stained tissues were in the lung (18.1 ± 3.1 cells/$0.2mm^2$).

Tissue	Toluidine blue (MC)	Carbol chromotrope (GL)	Anti-GMCP positive cells	Anti-human tryptase positive cells	µg GMCP per g wet weight tissue
Gastro-intestinal tissues					
Abomasum	6.7 (±2.7)	2.9 (±2.5)	2.8 (±2.1)	6 (±2.1)	0.2 (±0.09)
Duodenum	14.6 (±2.5)	7.6 (±1.8)	18.3 (±5.0)	7.7 (±2.2)[a,b]	8.2 (±1.3)
Jejunum	16.1 (±0.9)	10.1 (±1.8)	18.9 (±0.4)	8.7 (±3.4)[a,b]	7.8 (±1.4)
Ileum	10 (±1.4)	1.7 (±1.8)	9.8 (±1.3)	7.3 (±0.8)	0.6 (±0.08)
Colon	4.4 (±1.5)	0	4.1 (±2.6)	5 (±0.6)	0.1 (±0.02)
Mesenteric lymph node	13.5 (±3.5)	0	9.9 (±2.68)	11.2 (±3.0)	3.8 (±3.0)
Respiratory tissues					
Trachea	0	0	0.1 (±0.1)	0	0
Lung	18.1 (±5.4)	0	0.9 (±0.6)[a]	2.2 (±1.2)[a]	0.05 (±0.02)
Bronchial lymph node	16.5 (±3.0)	0	2.9 (±1.2)[a,b]	10.5 (±2.3)	0.3 (±0.1)
Other tissues					
Skin	12.6 (±2.7)	0	0.3 (±0.2)[a,b]	10.2 (±3.3)	0.04 (±0.03)
Liver	6.5 (±1.6)	0	0.1 (±0.1)[a]	0.8 (±0.5)[a]	3.29 (±1.2)
Spleen	4.8 (±0.8)	0	2.3 (±1.5)	2.3 (±0.8)	0.09 (±0.08)
Heart	1.8 (±0.5)	0	1.6 (±1.2)	0.6 (±0.4)[a]	0.05 (±0.04)

Table 4.1 Mean (±SD) results for tissue cell counts and tissue GMCP concentrations. Figures for intestinal tissue cell counts are expressed as per VCU. Figures for all other tissues are expressed as per $0.2mm^2$. Figures for GMCP ELISA are expressed as µg/g wet weight tissue. Superscripts denote significance, a = significantly fewer positive cells than the equivalent toluidine blue or total mast cell counts; b = significantly fewer positive cells than the equivalent anti-GMCP or anti-tryptase stained tissues. See text for p values.

There were fewer GMCP-positive cells in comparison to toluidine blue stained cells in the bronchial lymph node ($p < 0.005$; Fig. 4.4a and b), liver ($p < 0.005$), skin ($p < 0.005$; Fig 4.4d and e) and lung ($p < 0.01$) whilst, there were fewer anti-tryptase positive cells compared to total mast cells or toluidine blue stained cells in the duodenum ($p < 0.05$), jejunum ($p < 0.05$), liver ($p < 0.005$), heart ($p < 0.05$) and lung ($p < 0.005$). Comparisons between tissues stained with anti-GMCP and anti-tryptase also demonstrated significant differences in the numbers of positive cells in the duodenum ($p < 0.05$), jejunum ($p < 0.01$), bronchial lymph node ($p < 0.01$; Fig 4.4 b and c) and skin ($p < 0.01$; Fig. 4.4e and f) but not the lung or liver ($p > 0.05$). No statistically significant differences between the numbers of cells staining histochemically or immunohistochemically were observed in the other individual tissues examined.

Figure 4.1 shows that there was a strong positive correlation ($R^2 = 0.90$, $p < 0.0001$) between toluidine blue positive mast cell numbers and GMCP-positive cell counts from the gastro-intestinal tissues. This was slightly stronger than the correlation obtained between the total mast cell counts and the GMCP-positive cell counts ($R^2 = 0.88$, $p < 0.0001$) (not shown). The relationship was maintained when comparing toluidine blue positive mast cell numbers with anti-GMCP cell counts for all tissues except the respiratory tissues, liver and skin ($R^2 = 0.77$, $p < 0.0001$; Fig 4.2), but fell markedly ($R^2 = 0.27$, $p < 0.001$; Fig. 4.2), when counts from these tissues were included in the regression calculation.

In terms of tissue protease concentrations, both the duodenum and jejunum contained significantly more GMCP (8.2 ± 0.8 and 7.8 ± 0.8 μg/g $p < 0.05$; Table 4.1) than any other tissue except the mesenteric lymph node (3.8 ± 1.7 μg/g). Apart from gastro-intestinal tissues, the liver also contained relatively large amounts of GMCP (3.29 ± 0.7 μg/g).

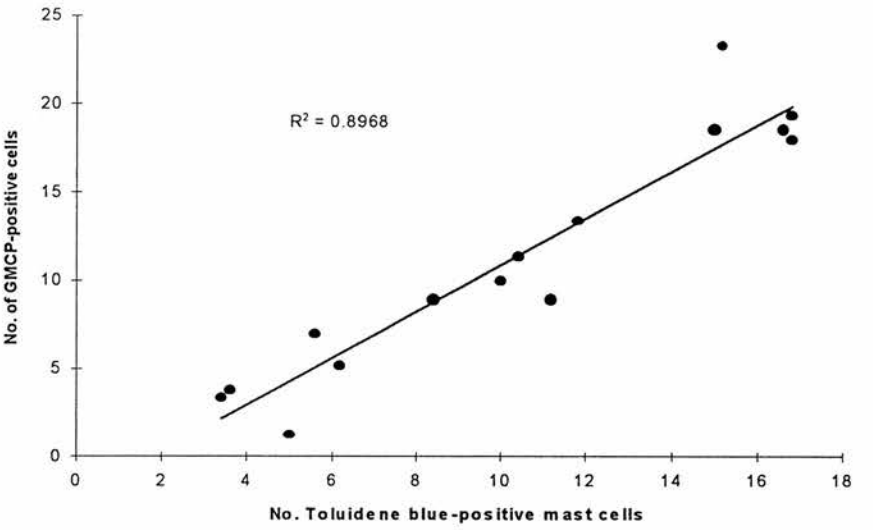


Fig. 4.1 Comparison of the numbers of toluidine blue stained mast cells and GMCP-positive mast cells in the gastrointestinal tract (abomasum, duodenum, jejunum, ileum and colon). Each point represents the counts for sequential sections from an individual tissue ($R^2 = 0.90$, $p < 0.0001$)

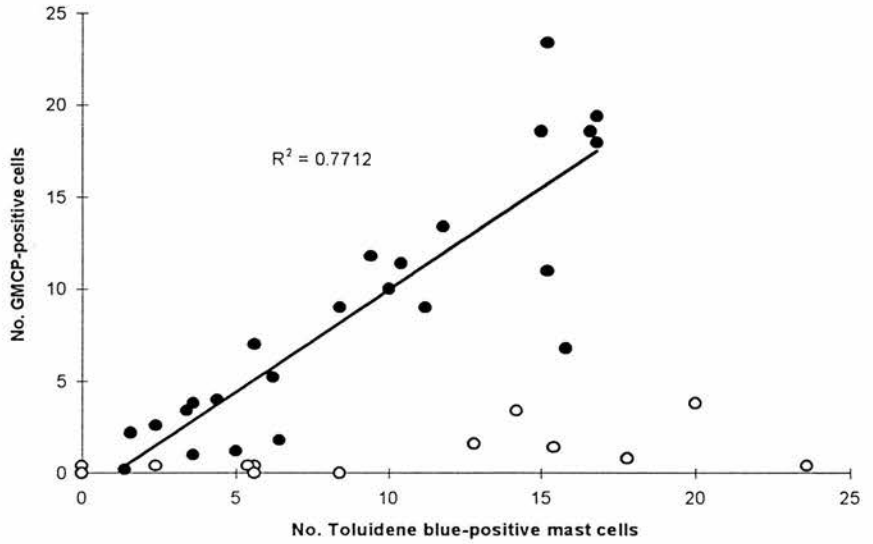


Fig. 4.2 Comparison of the numbers of toluidine blue stained mast cells and GMCP-positive mast cells in all tissues. Each point represents the counts for sequential sections from an individual tissue. The solid points (•) represent the counts for all tissues except the respiratory tissues, liver and skin ($R^2 = 0.77$, $p < 0.0001$). The clear points (◦) represent the counts from the respiratory tissues, liver and skin. When these tissues are included in the regression calculation, the correlation co-efficient drops to $R^2 = 0.27$; $p < 0.001$ (line not shown).

Figure 4.3a Anti-human tryptase positive cells in a goat jejunal villus. Note the similar anatomical distribution and morphology to cells reacting with polyclonal anti-GMCP in Chapter 3 (Fig. 3.8b). Note also the presence of a positive staining intra-epithelial GL. Tissue fixed in 4% paraformaldehyde (x 400).

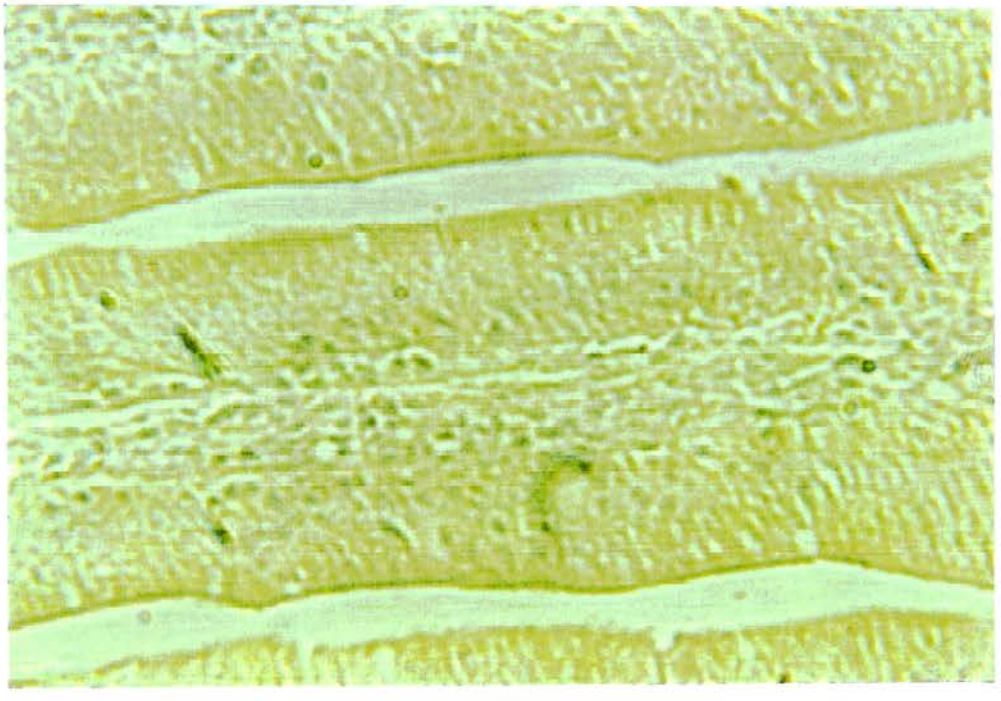
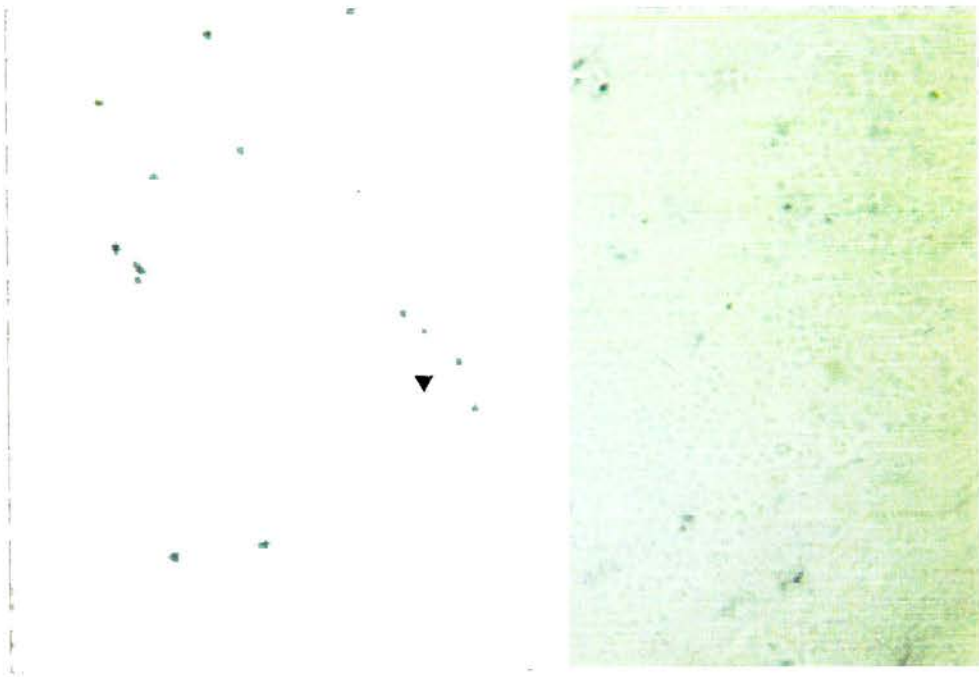


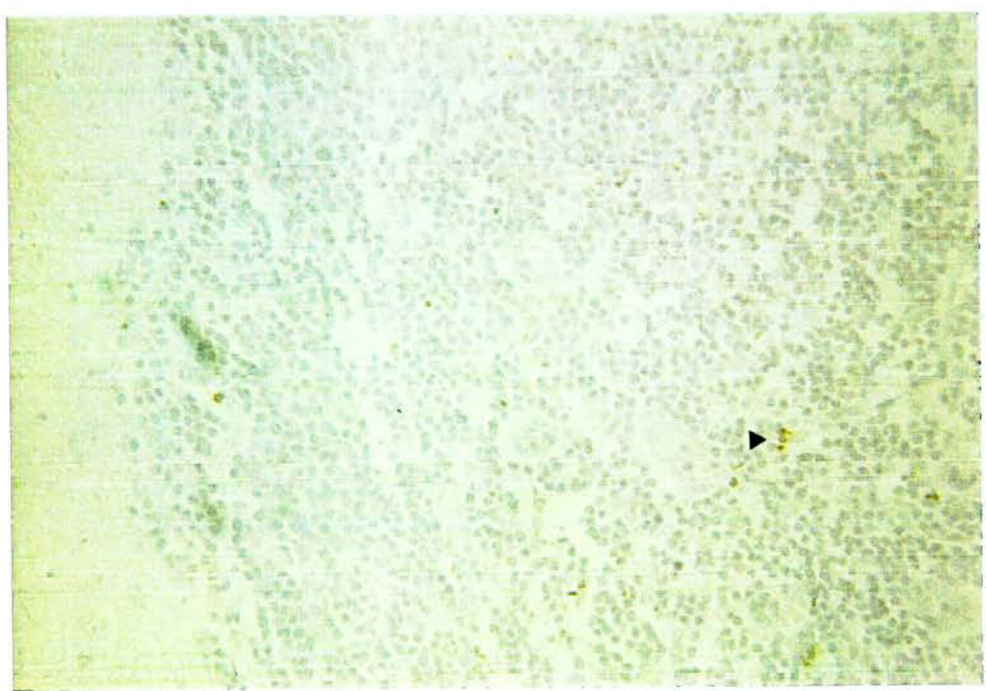
Figure 4.3b Negative control section incorporating normal rabbit serum diluted 1:500 in place of the anti-tryptase primary antibody. Tissue fixed in 4% paraformaldehyde (x 400).

Figure 4.4 (following pages) Goat bronchial lymph node (a, b and c) and dermis (d, e and f) stained with toluidine blue (a and d); anti-GMCP (b and e) and anti-human tryptase (c and f). Note the lack of cells staining positive in tissues incubated with anti-GMCP (arrows); compare with the numbers of faintly positive cells in tissues stained with toluidine blue (arrows) and and the numbers of strongly positive cells in tissues incubated with anti-human tryptase. Negative controls for the immuno-localised tissues incorporating normal rabbit serum diluted 1:500 in place of the primary antibody were all negative (not shown). 4% Paraformaldehyde fixed tissues (x 250).

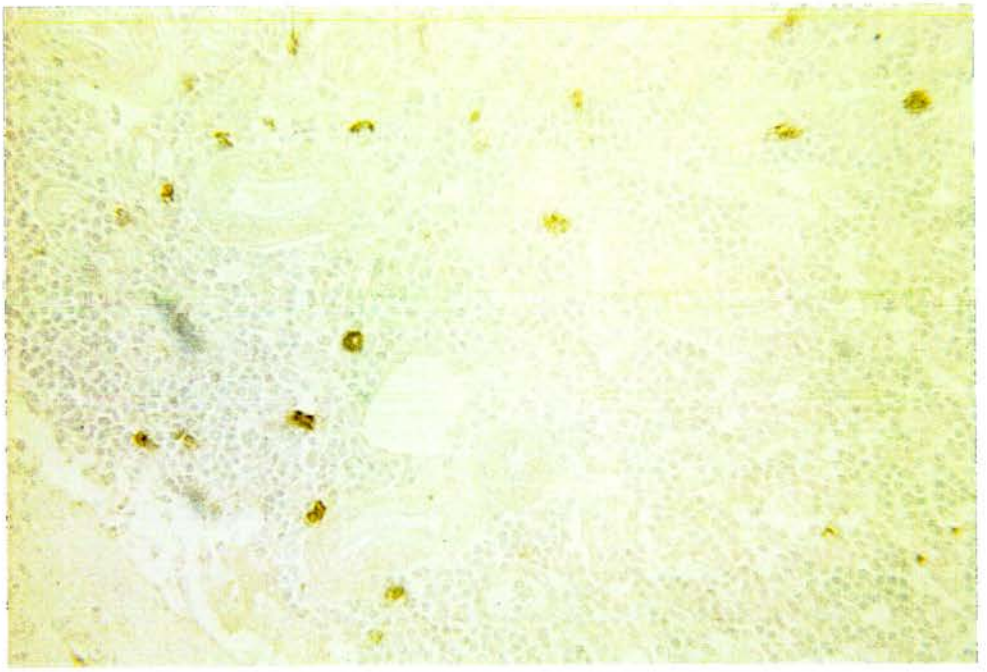
a



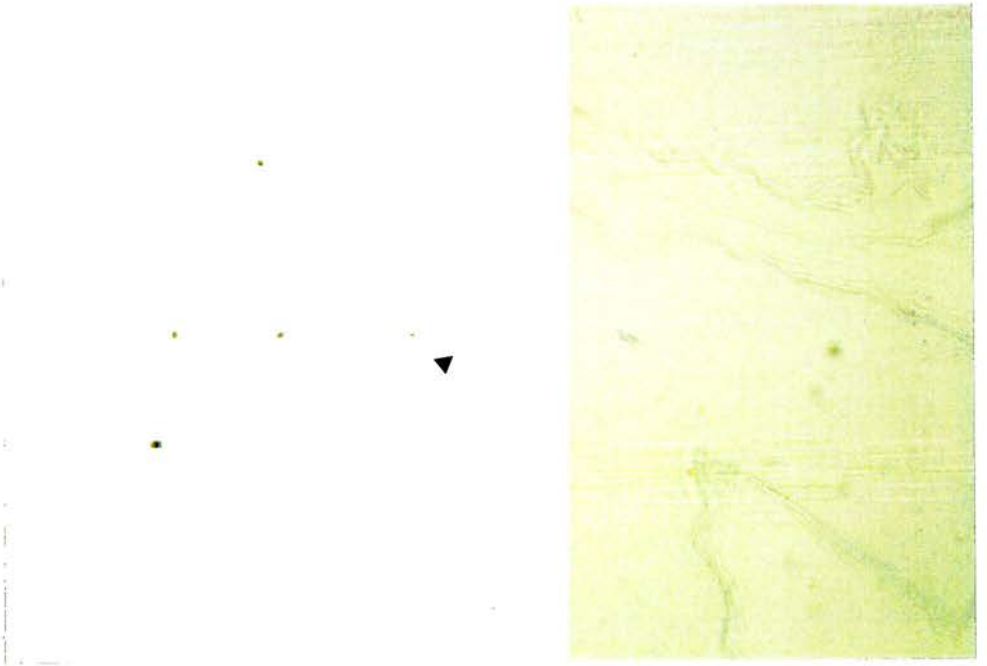
b



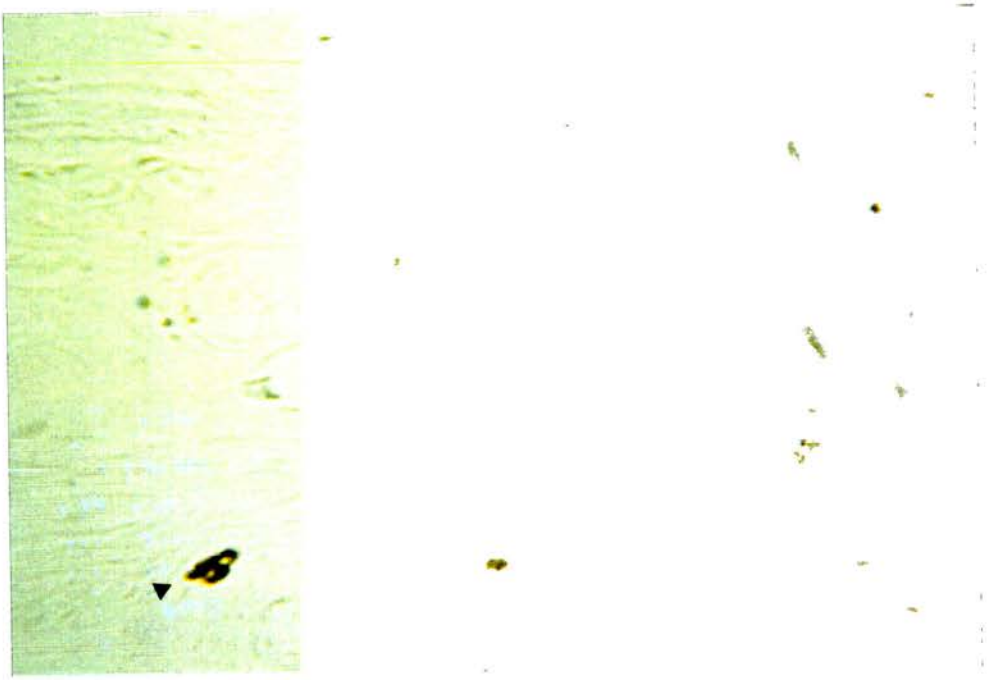
c



d



e



f

4.4 Discussion

Mast cells were detected with toluidine blue, polyclonal anti-GMCP antibody and anti-human tryptase antibody in all tissues examined except the trachea. In addition, GL could be detected histochemically in the gastro-intestinal tissues from the abomasum to the ileum with the highest numbers of GL relative to the total mast cell counts found in the abomasum, duodenum and jejunum. It is interesting to note, that these areas would have been exposed to the highest numbers of *T. circumcincta* and *T. vitrinus* during the challenge period prior to slaughter. Direct exposure to nematodes, may therefore influence the prevalence of this particular cell type in these tissues. Immunohistochemical staining also demonstrated that many GL contained GMCP and/or a putative goat tryptase enzyme, further suggesting that the cells are closely related to and derived from the same cell lineage as MMC (see also Chapter 3 and Chapter 8). Regression analysis demonstrated that the numbers of GMCP-positive cells correlated best with cell counts from histochemically stained tissues from the gastro-intestinal tract, although the relationship was slightly better for the toluidine blue counts for MMC ($R^2 = 0.90$) than the combined toluidine blue and carbol chromotrope (total mast cell) counts for both MMC and GL ($R^2 = 0.88$). This indicates that not all GL contained GMCP, although, as outlined in Chapter 3, many GL stained relatively faintly with anti-GMCP (Fig. 3.8 a and b). This possibly reflected their low mediator content, which would be expected for MMCs that had already degranulated (Huntley *et al.* 1984a). As a result, some poorly staining cells may have been missed when counting cells on sections stained with anti-GMCP.

In the lung and the liver, despite the presence of relatively high concentrations of GMCP, significantly more MMC were detected with toluidine blue than with anti-GMCP or anti-tryptase. The reasons behind this remain unclear, although it may indicate the presence of mast cells containing neither GMCP or tryptase, or a very sparse population of cells containing extremely high concentrations of GMCP per cell.

However, a more likely explanation is that the 4% paraformaldehyde /PBS fixative used to prepare the tissues blocked antibody binding in these sections. This problem has also been encountered with ovine lung parenchyma fixed in 10% formalin (D. Collie, personal communication). In this case, the tissue sections required treatment with 0.1% porcine trypsin prior to staining positively with the same batch of polyclonal anti-human tryptase. Given the prominence of tryptase reactivity in the goat bronchial lymph node tissues (Fig. 4.4c), it is possible that the goat lung parenchyma also contains substantial populations of tryptase staining cells. Further work is therefore required to optimize the fixation and/or staining conditions for antibody binding in these tissues.

The highest concentrations of GMCP were found in the duodenal and jejunal tissues. This is in agreement with the abomasal and jejunal tissue surveys carried out in Chapter 3. These tissues also had a higher concentration of GMCP calculated on a per GMCP-positive cell per VCU basis (0.44 and 0.41 μg/g/cell/VCU) than the ileum and colon (0.061 and 0.024 μg/g/cell/VCU). Unfortunately, due to the different counting method required for enumerating cells in the abomasum (cells/0.2mm^2 instead of cells/VCU), these values could not be related directly to the protease concentrations per cell per counting area in this tissue. Since the animals were infected with a mixed population of abomasal and small intestinal worms, the higher values for the upper small intestine may indicate increased upregulation and/or storage of GMCP in individual MMCs in response to nematode exposure. However, in the absence of worm-free control animals for comparison, this may also indicate that the mast cells in this area merely contain intrinsically higher levels of protease than mast cells from other areas of the intestine.

The linear regression results comparing counts for toluidine blue and GMCP-positive cells from different tissue sites indicate that GMCP is present in a high proportion of toluidine blue-positive cells in all tissues except the respiratory tissues,

liver and skin. The finding that relatively low numbers of cells stained for GMCP in the skin and liver was in agreement with the previous findings of Sture *et al.* (1996) for the distribution of SMCP in sheep. The current study also suggests that goat mast cells in the bronchial lymph node and possibly the lung are relatively deficient in GMCP, although as discussed above, immunoreactivity in the liver and lung may be poor due to problems with tissue fixation.

Although weak staining for GMCP was observed in the skin and bronchial lymph node, this was not demonstrated when the equivalent tissues were stained with anti-human tryptase antibody indicating that, in these tissues, the method of fixation was unlikely to be a problem. Here, significantly more cells with an equivalent morphology and anatomical position to the toluidine blue stained cells were positive after incubation with anti-tryptase than with anti-GMCP antibody (Fig 4.4 a-f). Accurate interpretation of results obtained using heterologous antibody is difficult, but the ability to stain the majority of mast cells in these tissues may indicate heterogeneity in terms of the distribution of GMCP and a putative goat mast cell tryptase. If so, these findings show that toluidine blue-positive cells in majority the of tissues examined contain chymase and/or tryptase. However, in the bronchial lymph nodes, skin and possibly the lung and liver, the majority of cells appear to contain tryptase with a minority of cells containing GMCP. In contrast, in the remaining tissues, including those in the gastro-intestinal tract, the majority of the cells appear to contain both GMCP and tryptase. At present, it is not possible to determine how GMCP and the putative goat mast cell tryptase enzyme are distibuted amongst mast cell populations in individual tissues. However, dual immunofluoresence studies could investigate this by using mouse monoclonal antibody to GMCP (Chapter 3) and the rabbit anti-human tryptase antibody. Future efforts should also be directed at the further characterisation of the putative goat mast cell tryptase enzyme. Such studies will be required to more fully define the enzyme's role *in vivo*, as well as enable the

development of homologous, immunospecific reagents for its detection and quantification.

CHAPTER 5

THE CLONING AND SEQUENCING OF cDNA FOR GMCP

5.1 Introduction

Revolutionary techniques in molecular biology have enabled researchers to characterise proteins through the interpretation and manipulation of genetic information that co-ordinates and encodes their synthesis. This has produced major insights into the regulatory, structural and functional properties of biological compounds in ways that are not feasible using conventional purification and biochemical techniques alone. The genomic and complementary DNA (cDNA) sequences for a number of mast cell neutral granule proteases have already been determined in a number of species (Table 5.1, page 146). These studies have proved particularly useful, allowing predictions to be made regarding molecular size, net charge, presence of potential glycosylation-sites and tertiary structure. This last point is particularly important with regard to enzymes, since in conjunction with computer modelling and crystallisation studies, such information allows detailed analyses of the enzyme's active site.

Active site conformation has important implications for defining the enzyme's catalytic activity and substrate specificity, which may be attributed to the shape and/or presence of certain charged amino acid residues within the substrate binding pocket. In this instance, it will be particularly relevant to examine this feature of GMCP and SMCP, since they have both been shown to exhibit an unusual dual substrate specificity (Chapter 3; Pemberton *et al.*, 1997a). Further information on the structural requirements for potential substrates may also help to define the role of these proteases *in vivo*.

In addition to predicting amino acid sequences, labelled cDNA probes may also be used to detect the presence of mRNA for proteases within tissues under investigation. In studies involving mast cell proteases, reverse transcriptase-polymerase chain reactions (RT-PCR) analysis may prove advantageous, enabling measurements to be made on the comparative levels of protease mRNA synthesis in

tissues rather than direct enzyme concentrations in tissues, serum or lymph by ELISA. This may circumvent the problem of obtaining artificially low ELISA results for mast cell proteases due to the presence of protease inhibitors (Huntley, 1991) or possible differences in the kinetics of protease synthesis, storage and release from MMC in both species (see Chapter 6).

Molecular probe techniques have also been used on rodent and murine RNA samples obtained *in vivo* from tissues as well as *in vitro* from BMMC and tranformed cell lines. They have been able detect the presence of transcripts for proteases that are either only transiently expressed during cell maturation or are not normally expressed as a protein product. These include the mouse tryptase enzyme MMCP-7 which was initially found only in early murine BMMC cultures (McNeil, Reynolds, Schiller, Ghildyal, Gurley and Stevens, 1992) and MMCP-L (a mouse mast cell like protease) which has yet to be detected as a protein despite having a high predicted amino-acid homology with other murine mast cell chymases (Serafin *et al.*, 1991). The finding that mast cells contain carefully regulated genomic information for synthesising a wide variety of proteases has stimulated recent studies examining the mechanisms controlling protease expression *in vivo* and *in vitro* (reviewed Springman and Serafin, 1995). These have been undertaken to examine the processes involved in producing phenotypic heterogeneity amongst mast cells *in vivo,* where the differences in protease expression of are an important distinguishing characteristic for certain mast cell subsets (reviewed, Huntley, 1992). Most results have now implicated cytokines, particularly those produced locally within the peripheral tissues, as being important for regulating protease transcription in mast cells undergoing the final stages of differentiation. However, the precise intra-cellular pathways and genomic regulatory elements involved are still largely unknown (reviewed Springman and Serafin, 1995). As yet, only one mast cell chymase has been isolated from the sheep (Huntley *et al.*, 1986) and the goat (Chapter 3) although immunohistochemical studies heve

demonstrated that heterogeneity in terms of mast cell protease content occurs in sheep (Sture *et al.* 1995) and the goat (Chapter 4). Therefore, it will be interesting to see if molecular analysis of sheep and goat mast cell DNA libraries also uncovers the presence of a family of genes coding for further proteases.

A further advantage of isolating genomic or cDNA for proteins expressed in low concentrations *in vivo*, is the ability to insert this DNA sequence into a bacterial or mammalian expression system, enabling the protein to be expressed in larger quantities *in vitro*. This approach may be relevant in the case of GMCP which is present in low concentrations in gastro-intestinal tissues *in vivo* (Chapter 3), making it difficult to obtain sufficient amounts for ELISA and biochemical characterisation studies. For the latter, the use of a mammalian expression system would probably be desirable to reproduce post-translational processing modifications that may occur as the translated protein acquires the correctly folded tertiary structure exhibited by the mature, active enzyme *in vivo*.

In summary, it can be seen that there are distinct advantages to be gained by unravelling the cDNA structure for GMCP. The aim of this Chapter is to achieve this by isolating RNA from caprine BMMC that have been grown *in vitro* using rOvIL-3 and rOvSCF. These cytokines have been shown to produce populations of ovine and caprine mast cells from bone marrow progenitor cells (Sture, 1996, Chapter 7). BMMC were chosen as a source material for two reasons; firstly, the presence of immature mast cells can be demonstrated as early as day 2 in the cultures and they increase rapidly in number to make up approximately 80% of the total cell population by day 16 (Chapter 7) and, secondly, these cells also express GMCP by day 2 (Chapter 7). The use of these cells should therefore maximise the chance of extracting GMCP specific mRNA from the cell pellet preparations.

The majority of the work described in this Chapter was performed at the Royal (Dick) School of Veterinary Studies (RDSVS), in the laboratories of Professor

Hugh Miller. Many of the primers and probes used for amplifying and blotting of the goat RNA and DNA samples were very kindly provided by Dr. S. Macaleese who was simultaneously working on sequencing the cDNA for SMCP. The results obtained here will therefore compare the sequences obtained for GMCP with those obtained for SMCP. This will provide further comparative data on the molecular characteristics of mast cell proteases present in these two species.

Mast Cell Proteases	References
Chymases	
Mouse mast cell protease-1 (MMCP-1)	Huang, Blom and Hellman, 1991*
Mouse mast cell protease-2 (MMCP-2)	Serafin, Reynolds, Rogelj, Lane, Conder, Johnson, Austen and Stevens, 1990
Mouse mast cell protease-4 (MMCP-4)	Serafin, Sullivan, Conder, Ebrahimi, Marcham, Johnson,Austen, and Reynolds, 1991*
Mouse mast cell protease-5 (MMCP-5)	McNeil, Austen, Somerville, Gurish and Stevens, 1991*
Mouse mast cell protease-L (MMCP-L)	Serafin, Reynolds, Rogelj, Lane, Conder, Johnson, Austen and Stevens, 1990
Human chymase	Caughey, Zerweck, and Vanderslice, 1991*
Rat mast cell protease II (RMCP II)	Sarid, Benfey and Leder, 1989*
Dog chymase	Caughey, Raymond and Vanderslice, 1990*
Tryptases	
Mouse mast cell protease-6 (MMCP-6)	Reynolds, Gurley, Austen and Serafin, 1991
Mouse mast cell protease-7 (MMCP-7)	McNeil, Reynolds, Schiller, Ghildyal, Gurley, Austen and Stevens, 1992
Human tryptase-α	Miller, Westin and Schwartz, 1989
Human tryptase-β	Vanderslice, Craik, Nadel and Caughey, 1989
Human tryptases I, II and III	Vanderslice, Ballinger, Tam, Goldstein, Craik and Caughey, 1990
Dog tryptase	Vanderslice, Craik, Nadel and Caughey, 1989

Table 5.1 References for selected mast cell serine esterase enzymes from a variety of species whose genomic or cDNA sequences have now been determined. *chymase sequence data used for the initial construction of SMCP primers based upon areas of consensus between the species

5.2 Mapping of oligonucleotide sequences used for priming and blotting

Figure 5.1 represents an entire cDNA sequence obtained for SMCP cDNA showing the positions of oligonucleotide sequences (coloured letters) used as primers for amplifying the goat cDNA templates, as well as for hybridising to check the specificity of the resulting PCR products. Primers P1, P2, P3 and P4 were initially synthesised by J. Handley at the RDSVS, based upon areas of consensus in previously published cDNA sequences for rodent (RMCP II), murine (MMCPs 1, 4 and 5), canine and human mast cell chymases (Sarid *et al.*, 1989, Huang *et al.*, 1991; Serafin *et al.*, 1991; McNeil *et al.*, 1991; Caughey *et al.* 1990 and Caughey *et al.* 1991) (Table 5.1). In addition, a number of degenerate primers (not shown) were also synthesised from the NH_2-terminal amino acid sequences published for isolated SMCP (Pemberton *et al* 1997a) and bovine duodenase (Zamolodchikova *et al.* 1995b). All subsequent primers were synthesised on the basis of results obtained from sequencing cloned SMCP and GMCP cDNA fragments. Also depicted in Fig. 5.1 are the positions of the start and stop codons indicating the start of the signal peptide sequence and the end of the mature protein sequence. The position of nucleotides encoding the pro-peptide G-E residues associated with an inactivated form of the enzyme which exists prior to activation and storage within the mast cell granule (Urata, Karnik, Graham and Husain, 1993) are also shown. This dipeptide is positioned immediately before the start of the mature enzyme peptide sequence which possesses the classical I-I-G-G tetra-peptide sequence found in the majority of chymase enzymes (Springman and Serafin, 1995).

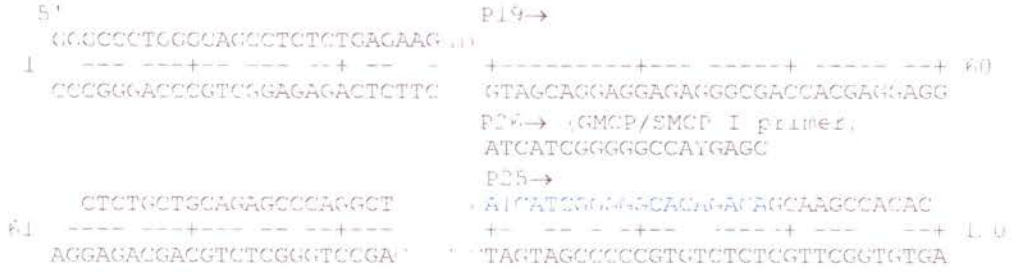


Fig. 5.1 Complete cDNA sequence for SMCP II showing the positions of the primers used for amplifying and probing the GMCP DNA fragments. The positions of the start and stop codons are shown in red, along with the nucleotide sequence present at the start of the inactivated SMCP pro-enzyme and the 3' polyA tail. Also shown are the positions of 2 introns present in the initial P3 - P9 central fragment obtained for GMCP (see text).

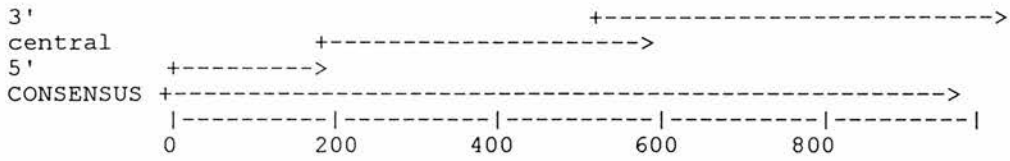


Fig. 5.2 Fragment assembly of GMCPII sequence data showing the positions of the amplified GMCP II cDNA fragments relative to one another. Sequences compared with a GMCPII clone (consensus) containing the entire GMCP II cDNA sequence.

5.3 Sequencing strategy

This was designed to follow closely the successful strategy used previously for the isolation of a cDNA sequence for SMCP (S.M.Macaleese, personal communication), and initially involved sequencing the GMCP cDNA as 3 separate fragments. The first fragment, termed the central fragment, was isolated to give a sequence for the region lying between the P3 and P9 primers. This was followed by a 3' fragment covering the region between P8 and RACEdT primers and finally a 5' fragment covering the areas between P15 primer and the signal peptide sequence (Figs. 5.1 and 5.2). Once the sequences of these 3 fragments were checked for homology with sequences obtained for SMCP, primers specific for the 5' and 3' ends were constructed to allow the production of amplified clones containing the entire GMCP sequence. This complete sequence was then used to confirm the position and content of the initially obtained sequence fragments (Fig 5.2), as well as to provide a whole enzyme cDNA probe for further cDNA hybridisation or tissue mRNA hybridisation (Northern blotting) studies.

5.4 Results

5.4.1 Isolation of RNA

Caprine bone marrow cells for mRNA extraction were harvested from cultures fed with optimal concentrations of recombinant ovine interleukin-3 (rOvIL-3) only, or rOvIL-3 and recombinant ovine stem cell factor (rOvSCF), as outlined in Chapter 7.

Cells (5 x 10^6), were removed from each culture on day 2, day 5 and day 7 and lysed in 1ml Tri-reagent (Sigma Cat. No. T 9424) before storing at -70 ^{o}C. The samples were subsequently processed to extract the total RNA as outlined in Chapter 2.7.1. Samples from the isolated preparations were separated on a formaldehyde gel to check the integrity of the RNA (Fig. 5.3). Cells grown in rOvIL-3 alone and harvested at day 7 contained significantly more total RNA than the other samples (Fig. 5.3; lane 3). Therefore, this sample was chosen for construction of a cDNA library by following the reverse transcription (RT) protocol for the central and 3' GMCP fragments using a random hexamer primer as outlined in 2.7.4. All subsequent polymerase chain reaction (PCR) amplification steps were carried out as described in Chapter 2.7.5.

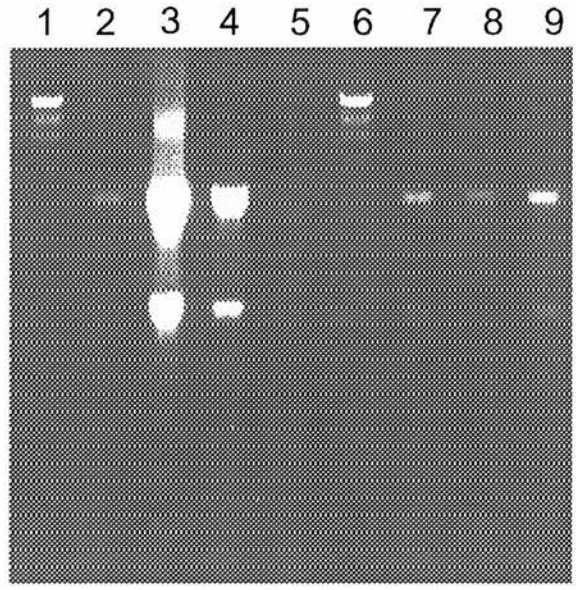


Fig. 5.3 Formaldehyde gel of total RNA preparations from goat BMMC. Lanes 1 and 6 RNA standards. Lanes 2, 3 and 4; day2, day7 and day 12 BMMC grown in rOvIL-3 only. Lanes 7, 8 and 9; day 2, day7 and day12 BMMC grown in rOvIL-3 and rOvSCF.

5.4.2 Cloning the central fragment of GMCP DNA

Products from the RT reaction were initially amplified in the presence of ovine P1 and P5 primers. However when these were separated on a 0.8% agarose gel bands were not evident in any of the samples. The primary PCR products were therefore re-amplified using P3 and P9 which both lie internal to P1 and P5 (Fig. 5.1). This time, a 1.5 kilobase pair (kb) band was present (Fig 5.4 lanes 2 - 4), indicating that a second amplification step had been required to increase the DNA concentration of the primary PCR products to levels sufficient for visual detection. An agarose gel containing samples of this secondary PCR product was Southern blotted and probed with a digoxigenin labelled P4 (P4-dig) probe (Fig. 5.1), to test whether the fragment contained the correct sequence for a mast cell protease. The blotted sample gave a positive 1.5kb band (Fig 5.5 lane 2) and aliquots of the secondary PCR product were ligated into an Invitrogen 3.9kb pCR™2.1 plasmid before transforming into *E. coli* for cloning as outlined in Chapter 2.7.11. After purification and restriction enzyme digestion of the cloned plasmid DNA (see 2.7.12 and 2.7.13), the digested samples were separated on a 0.8% agarose gel, Southern blotted and again re-probed with P4-dig to check that the cloned plasmids contained the correct DNA inserts. The EcoRI digest product representing the PCR product removed from the plasmid gave positive 1.5 kb bands for 3 clones. DNA quantification was performed on these samples (see 2.7.14), and an aliquot was sent for sequencing.

The size of the cloned band (1.5kb) was approximately 3 times larger than that predicted from the equivalent 445 bp P3 to P9 fragment isolated from SMCP (Fig. 5.1) indicating that the clone was probably the amplified product of either unprocessed mRNA or genomic DNA, and not cDNA. This was confirmed when the forward and reverse analysis of the GMCP clone demonstrated the presence of two introns in the cloned fragment at the sites shown on Fig. 5.1. The first intron was so large, that its entire sequence was not determined and therefore it's size remains

unknown. However, the second intron found in the sequence from the reverse primer was shorter, containing only 375 bp before the coding region of the neighbouring exon could again be detected.

The potential presence of contaminating heteronuclear DNA in the cell total RNA preparations and the subsequent RT reaction products indicated that, in order generate a cDNA sequence for GMCP, the mRNA component would have to be purified from the total RNA. This was achieved by fractionating total RNA samples on a commercial oligodT cellulose column (see Chapter 2.7.2), which selectively binds the 3' polyA sequences present in mRNA. The integrity of the eluted mRNA fraction was checked on a formaldehyde gel, before repeating the RT reaction using a RACEdT primer in place of the previously used random hexamer primer. This primer also selectively binds to polyA mRNA sequences, ensuring that they undergo preferential reverse-transcription to produce maximal amounts of cDNA, with minimal genomic DNA contamination. These mRNA selected RT products were amplified twice using the ovine P1 and P9 primers used above, followed by two new primers constructed from the first goat DNA sequence results termed GP1 and GP2, which are found internal to P3 and P9 (Fig. 5.1). After the second amplification, a 400 bp band could be detected on a 0.8% agarose gel (Fig. 5.4 lane 7), indicating that it was the correct size for the required cDNA sequence based upon predictions from the previously obtained SMCP sequence (Fig. 5.1). This was confirmed by Southern blotting and probing with P4-Dig, which also produced a strong band at 400 bp (Fig. 5.5 lane 4). The amplified sample was ligated into the Invitrogen 3.9kb pCR™2.1 plasmid and cloned as described previously. Following restriction enzyme digestion of the plasmid DNA, blotted samples were again probed with P4-dig to confirm the presence of the required 400 bp cDNA inserts and positive samples were sent for sequencing. The results confirmed the presence of a 398 bp fragment without the intron sequences found in the previous samples. This fragment was aligned with the

previously obtained equivalent SMCP cDNA sequence using the GCG sequence analysis software (Chapter 2.14.2) and demonstrated a 94.3 % homology between the two sequences.

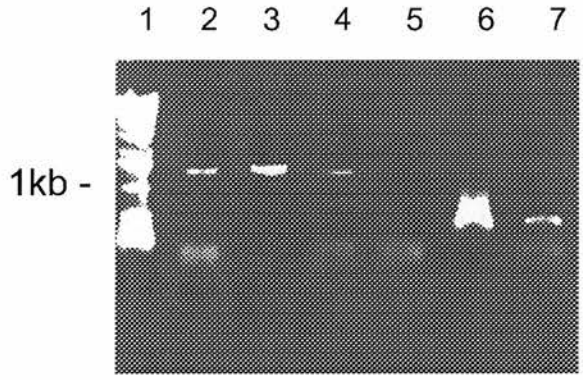


Fig. 5.4 0.8% agarose gel of PCR amplification products for the central fragment of GMCP II. Lane 1; DNA standards. Lanes 2,3 and 4; second PCR amplification from the total RNA RT reaction product showing the 1.5kb central fragment. Lane 6; first PCR from the mRNA selected RT reaction product. Lane 7; second PCR amplification from the mRNA selected reaction product showing the 400bp cDNA fragments.

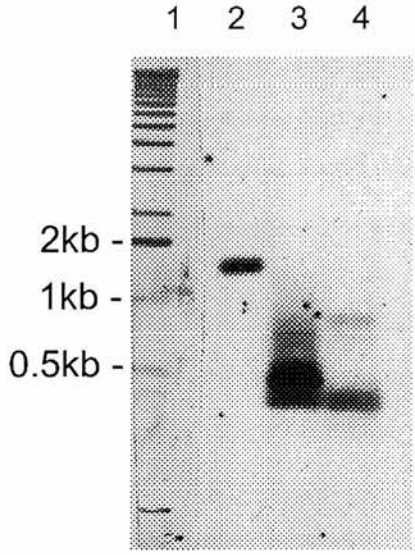


Fig. 5.5 Southern blot of the 1.5kb and mRNA selected PCR products for GMCP II probed with P4-dig. Lane 1; DNA standards. Lane 2; 1.5kb central fragment. Lane 3; primary amplification of mRNA selected RT product using P1 and P9. Lane 4; secondary amplification of the P1 and P9 amplification products using GP1 and GP2 showing a 400bp cDNA fragment which was cloned and sequenced.

5.4.3 Cloning of the 3' fragment of GMCP cDNA

The mRNA selected RT products prepared above, were amplified twice using P11 and RACEdT followed by P8 and RACEdT (Fig 5.1). This was designed to amplify selectively the 3' region of the GMCP cDNA present in the RT product. Following primary amplification, faint smeared bands were present on a 0.8% agarose gel. However, after re-amplification with P8 and RACEdT a strong band of approximately 400bp was observed which also reacted positively following Southern blotting and probing with a digoxigenin-labelled P9 probe (P9-dig). The sample was ligated and cloned as previously, and the resulting restriction enzyme digests of the purified plasmid DNA again blotted and re-probed with P9-dig to check for positive insertions of GMCP 3' cDNA (Fig. 5.6). Samples with positive inserts sent for sequencing were returned as a 440bp sequence which, on alignment, overlapped with the central GMCP cDNA fragment and showed 96.5% identity with the SMCP cDNA sequence.

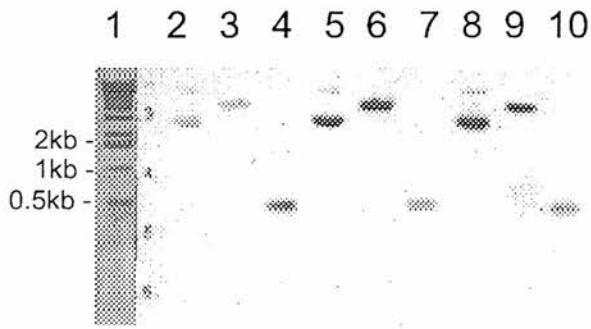


Fig. 5.6 Southern blot of restriction enzyme digests of cloned and plasmid ligated 3' GMCP II cDNA fragments probed with P9-dig. Lane 1; DNA standards. Lanes 2, 5 and 8; undigested plasmid. Lanes 3, 6 and 9; NotI linearised plasmids. Lanes 4, 7 and 10; EcoRI digested plasmids showing the free 400bp 3' cDNA inserts.

5.4.4 Cloning of the 5' fragment of GMCP cDNA

PCR amplification of the 5' segment of cDNA was hampered by the lack of primers specific for the 5' non-coding signal peptide sequences which lie upstream of the regions that encode the mature enzyme. A number of primers have already been synthesised on the basis of NH_2-terminal amino acid sequences obtained from samples of isolated SMCP and bovine duodenase. However, these were unable to amplify fragments from the ovine cDNA libraries, possibly as a result of their inherent degeneracy (S. Macaleese, personal communication). In any case, the use of primers based on the NH_2-terminal mature protein sequences alone would not allow the production of amplified clones containing signal peptide sequences which may be of importance in dictating the final cellular destination of the translated enzyme.

This problem was overcome by artificially tailing of the 5' ends of the mRNA selected RT preparations with polyA sequences, before amplifying them in a similar way to the naturally polyA-tailed 3' cDNA sequences. The poly A tailing reaction was carried out out using a commercial 5'/3' RACE (Rapid Amplification of cDNA Ends) kit (Boerhinger Cat. No. 1 734 792), according to the manufacturer's instructions. The kit involves an additional cDNA purification step for the RT products before tailing the 5' ends of the resulting cDNA fragments with polyA sequences using terminal transferase. The tailed sequences were then amplified twice using a special oligodT anchor primer provided in the kit, which is functionally identical to the RACEdT primer used previously for RT reactions and the 3' fragment amplifications. In the first amplification, P14 was used with the oligo dT anchor primer, resulting in the production of a faint 225kb band. The sample was re-amplified using P15 and oligo dT which produced a thick smear of bands with the strongest lying in the 100-200kb range. The gel was blotted and probed with digoxigenin labelled P12 (P12-dig) which produced thick bands for both the primary and secondary amplification

products at approximately 225kb and 200kb respectively. The secondary amplification product was ligated, cloned and sequenced as previously, returning a 185bp fragment that demonstrated a 91.1% identity with the SMCP cDNA sequence (Fig. 5.7).

5.4.5 The production of a full length cDNA clone for GMCP

After the sequence results obtained from the GMCP cDNA fragments, it was necessary to produce a full length GMCP cDNA clone to confirm the relative positions of the GMCP cDNA fragments as well as their nucleotide sequences. This was achieved using a combination of a P19 primer developed from a 5' signal sequence obtained for SMCP, along with P20 and P22 primers which are specific for the areas that incorporate and lie external to the SMCP cDNA stop codon (Fig. 5.1). The mRNA selected RT products were amplified initially with P19 and P22 followed by P19 and P20. The second PCR amplification produced a band of approximately 700bp which was ligated and cloned as described previously. Clones were selected by probing Southern blots of EcoRI digested plasmid DNA with P12-dig before sending for sequencing. The sequence results showed that the cDNA insert contained 714 bp, which was 37 bp less than the equivalent SMCP cDNA sequence. When the sequences were aligned with the concatenated GMCP cDNA fragments, it could be seen that this deficit was due to a loss of nucleotides between C^{137} and T^{177} (Fig. 5.7). Despite this, the cloned fragment still showed a 92 % identity with the SMCP cDNA and an 87 % identity with the concatenated GMCP cDNA fragments. The identity between the full length GMCP cDNA clone and the combined GMCP cDNA fragments was lower than that shown between the full length GMCP cDNA and the SMCP cDNA sequences, due to marked differences in the signal peptide coding sequences which lie upstream from the G-E propeptide coding region (Fig. 5.7).

Fig. 5.7 Comparison of the 5' nucleotide sequences for the full length GMCP II (GMCP2) clone, SMCP II (smcp2) and the 5' GMCP II cDNA fragment (gmcp2con) The position of the start codon is shown in red. The markedly different signal peptide sequence obtained as a result of the amplification with the oligodT primer is shown in bold type. Note also the missing nucleotides in the GMCP II sequence shown as red dots.

5.4.6 Protein translations of the GMCP and SMCP cDNA sequences

The nucleotide sequences obtained for SMCP cDNA, the whole GMCP cDNA clone and the GMCP cDNA fragments were translated in the 5' to 3' direction for the three possible forward reading frames. In all three sequences, a G-E pro-peptide followed by the I-I-G-G tetra-peptide was noted for one of the reading frames. This reading frame was also in phase with the ATG start codons present in the SMCP cDNA and whole GMCP cDNA sequences (Fig 5.7), indicating that the resulting translation was correct for the signal peptide sequence as well as the pro- and mature enzyme amino acid sequences. The protein translations were aligned to one another and it was found that the GMCP full length clone exhibited 97% and

96.7% identity with the SMCP cDNA and the combined GMCP fragment cDNA translations respectively (Fig. 5.8). However, when these sequences were compared with the NH_2-terminal amino acid sequences for isolated SMCP (Pemberton *et al.*, 1997a), isolated GMCP (Chapter 3) and the complete amino acid sequence for isolated bovine duodenase (Zamolodchikova *et al.* 1995b) it was found that they exhibited much less homology. This included an overall identity of only 53 % occurring between the whole deduced GMCP peptide sequence, and the whole isolated bovine duodenase peptide sequence (Fig. 5.8).

This indicated that the cDNA sequences isolated for SMCP and GMCP may code for separate, albeit related, proteins to those isolated from ruminant intestinal mast cells *in vivo*. On the basis of this, these potentially novel proteases were designated as SMCP II and GMCP II and an alternative strategy was initiated to isolate and clone cDNA sequences coding for their isolated intestinal equivalents now termed SMCP I and GMCP I.

```
          1                                                    50
   gmcp2  GEIIGGTESK PHSRPYM... ......... ACGGFLIRRD FVLTAAHCAG
   smcp2  GEIIGGTESK PHSRPYMAYL EIVTSQGKQV ACGGFLIRRD FVLTAAHCAG
gmcp2con  GEIIGGTESK PHSCPYMAYL EIVTSREKQV ACGGFLIRRD FVLTAAHCAG
Bov duod    IIGGHEAK PHSRPYMAFL LFKTS.GKSH ICGGFLVRED FVLTAAHCLG
Isol SMCP   IIGGHEAK PHSRPYMAFL QFKISGKSYR FG
Isol GMCP   IIGG?EAK P?S?PYMAFL

          51                                                   100
   gmcp2  RSVTVTLGAH NIQKKEDTWQ RLEVIKQFPY PKYEPAGL.H DIMLLKLEEK
   smcp2  RSVTVTLGAH NIQKKEDTWQ RLEVIKQFPY PKYEPVGV.H DIMLLKLKEK
gmcp2con  RSVTVTFGAQ NIQK?EDTWQ RIEVIKQFPY AKYDPVGL.H DIMLLKLEEK
Bov duod  .SI VTLGAH NIMERERTQQ VIPVRRPIPH PDYNDETLAN DIMLLKLTRK

          101                                                  150
   gmcp2  A LTLAVGTL PLPPHVTFIR PGRMCQVAGW GRTGVKEPAS STLQEVKLRL
   smcp2  A LTLAVGTL PLPPHVTFIR PGRMCQVAGW GRTGVKEPAS STLQEVKLRL
gmcp2con  A LTLAVRTL PLLPPVTFIC PGRMCQVAGW GRTGVKEPAS STLQEVKLRL
Bov duod  ADITDKVSPI NLPRSLAEVK PGMMCSVAGW GRLGVNMPST DKLQEVDLEV

          151                                 165          200
   gmcp2  M.EPRACSHF PAFDHNLQLC VGNPQSTKTA FKGDSGGPLL CAGVAQGIVS
   smcp2  M.EPRACRHF RAFDHNLQLC VGNPQSTK.A FKGDSGGPLL CAGVAQGIVS
gmcp2con  M.EPRACSHF PAFDHNLQLC VGNPQSTK.A FKGDSGGPLL CAGVAQGIVS
Bov duod  QSEEKCIARF KNYIPFTQIC AGDPSKRRIS FSGDSGGPLV CNGVAQGIVS
                                                  ← P2

          201      2
   gmcp2  YGLFSAKPP  VFTRISPYRP WIDEVLKEN*
   smcp2  YGLSSAKPP. VFTRISPYRP WIDEVLKEN*
gmcp2frag YGLFSAKPPA VFTRISPYRP WIDEVLKEN*
Bov duod  YGKNDGTTPT VYTRISSFLP WIKRVMYLFK
```

Fig. 5.8 Deduced amino acid sequences from the SMCP II (smcp2) and GMCP II (gmcp2 and gmcp2con) cDNA sequences compared with NH_2-terminal amino acid sequences obtained from isolated SMCP (Isol SMCP) and GMCP (Isol GMCP) as well as the whole amino acid sequence for isolated bovine duodenase (Bov Duod) (all shown in standard single letter notation). Note the high degree of identity between the deduced amino acid sequences for SMCP and GMCP and their poorer identity with the amino acid sequences from the isolated proteases. ? = undetermined residue; * = stop codon site.; green residues = potential glycosylation sites. The catalytic triad of His, Asp and Ser residues are shown in blue whilst the S_1 active site residues are shown in red. The standard chymotrypsinogen numbered positions of these residues are shown above in red. Note also the position of P2 which shows consensus with GMCP II, SMCP II and bovine duodenase.

5.4.7 Isolation and cloning of a cDNA sequence for GMCP I

This was achieved using codon usage information from the SMCP II and GMCP II nucleotide sequences to synthesise a primer specific for the homologous

residues in the isolated NH_2-terminal sequences for GMCP I and SMCP I as well as by examining the ability of a full length GMCP II probe to hybridise with the resulting blotted PCR products under different conditions of stringency. The NH_2-terminus primer (P26, Fig. 5.1) was synthesised to match the nucleotides coding for the I-I-G-G region of GMCP II, but in place of the nucleotides coding for the subsequent T residue, a degenerate codon for the H residue found in the isolated SMCP I and bovine duodenase NH_2-terminus sequences was inserted (Fig 5.9). This was followed by a codon for the E residue that matched the codons found in the GMCP II and SMCP II sequences and finally two thirds of a codon for the subsequent A residue found in GMCP I, SMCP I and duodenase, thus avoiding the degenerate 3rd position which could have contained any of the four possible nucleotides.

GMCP II cDNA sequence	ATC	ATC	GGG	GGC	ACA	GAG	AGC
Deduced GMCP II amino acid sequence	I	I	G	G	T	E	S
Isolated SMCP amino acid sequence	I	I	G	G	H	E	A
Isolated GMCP amino acid sequence	I	I	G	G	?	E	A
Bovine duodenase amino acid sequence	I	I	G	G	H	E	A
NH_2-terminus primer (P26)	ATC	ATC	GGG	GGC	**CAY**	GAG	**GC***

Fig. 5.9 Construction of the P26 primer for selectively amplifying cDNA that codes for the NH_2-terminal amino acids of SMCP I and GMCP I. Primer sequence information is gained largely from the GMCP II and SMCP II nucleotide sequences but a degenerate codon for the H residue is placed after the codon for the second G residue. Here, the primer mixture contains an equal number of T and C nucleotides at the third position (represented here as a **Y**) since either combination can code for H. The last nucleotide has also been left out of the final codon (*) since it could contain any of the four possible nucleotides at this position and still code for an A residue.

The P26 primer was used in combination with P2 which is specific for an area of sequence showing 100% amino acid identity between GMCP II and bovine

duodenase (Fig. 5.1 and Fig. 5.8). Following primary amplification of the same mRNA selected RT products used to isolate GMCP II, a sample of the resulting PCR product was separated on a 0.8% agarose gel where it produced a strong band at approximately 550bp. The gel, which also contained secondary PCR products for GMCP II amplified using primers for the full length cDNA (P 19 to P20), as well as a truncated product using P25 (specific for the N-terminus of GMCP II, see Fig. 5.1) to P2 was blotted as previously. The blot was hybridized with a digoxigenin labelled probe made from full length (P19 to P20) cDNA for GMCP II (GMCP II-dig) under conditions of low and high temperature and washing stringency (see 2.7.10). This acts to alter the binding or annealing capacity of the GMCP II-dig probe to the blotted cDNA, thus highlighting differences in the sequence identity of cDNA sequences coding for related proteins. Under conditions of low temperature and washing stringency, (50 ^{o}C; 2 x SSC, 0.5 x SSC), the GMCP II-dig probe bound to the 714bp full length GMCP II cDNA band, as well as to the 550bp P25 to P2 truncated GMCP II and P26 to P2 potential GMCP I cDNA bands (Fig. 5.10a). After stripping the blot (see Chapter 2.7.9), and re-probing with GMCP-II-dig under conditions of high temperature and washing stringency (60 ^{o}C; 2 x SSC, 0.2 x SSC), probe binding to the potential GMCP I band was abolished whilst the GMCP II bands were almost as strong as previously (Fig. 5.10b). This result indicated significant differences in the sequence identity of the two PCR products which would be consistent with the low identity shown by the deduced amino acid sequences for GMCP II and bovine duodenase. The P26 to P2 PCR product was cloned and sequenced, producing a 552bp band that had 94.6% identity with an equivalent SMCP I cDNA clone amplified using the same primers (S. Macaleese, personal communication). In contrast, the nucleotide sequence for GMCP I has an identity of only 58.8% with GMCP II and 57.1 % with SMCP II (Fig. 5.11). The nominal peptide sequence for GMCP I was deduced, producing a sequence that shares 93.3 % identity with the

NH_2-terminal amino acid sequence for isolated SMCP I, at least an 83.3 % identity with the NH_2-terminal amino acid sequence for isolated GMCP I and 90.8 % identity with isolated bovine duodenase (Fig. 5.12). In contrast, there was only 65.1% and 64.6% identity with the deduced peptide sequences for GMCP II and SMCP II respectively.

Unfortunately, due to time constraints, it was not possible to continue the sequencing of GMCP I beyond the P2 primer to ascertain the 3' coding and non-coding regions of the enzyme. However, this has recently been achieved for SMCP using primers specific to SMCP I which lie internal to P2 and the RACE dT primer (S. Macaleese, personal, communication) (Fig. 5.11). The deduced nominal peptide sequence obtained from these clones demonstrates an 89.8 % identity with the peptide sequence for bovine duodenase (Fig. 5.12).

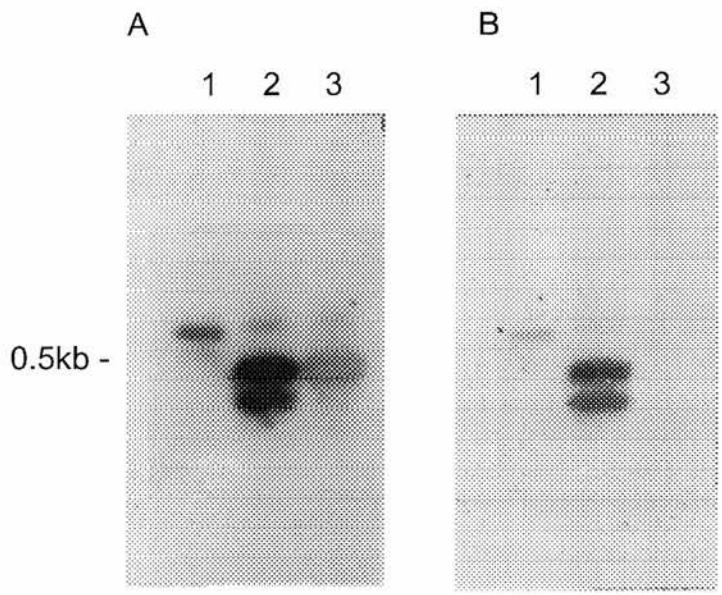


Fig. 5.10 a and b Southern blot of full length (P19 to P 20) and truncated (P25 to P2) GMCP II secondary PCR products (lanes 1 and 2) and GMCP I first PCR product (Lane 3) probed with GMCP II (P19 to P20)-dig under low (A) and high (B) stringency conditions. Note the absence of the GMCPI band in blot B.

```
1                                                                   50
gmcp2  .......... .......... ......ATGC ATCGTCCTCC TCTCCCGCTG
smcp2  GGGCCCTGGG CAGCCTCTCT GAGAAGATGC ATCGTCCTCC TCTCCCGCTG
gmcp1  .......... .......... .......... .......... ..........
smcp1  .......... .......... .......... .......... ..........
       51                                                  100
                                                     I   I  G  G
gmcp2  GTGCTCCTCC TCCTCTGCTG CAGAGCCCAG GCTGGGGAGA TCATCGGGGG
smcp2  GTGCTCCTCC TCCTCTGCTG CAGAGCCCAG GCTGGGGAGA TCATCGGGGG
gmcp1  .......... .......... .......... .........A TCATCGGGGG
smcp1  .......... .......... .......... .........A TCATCGGGGG
       101                                                 150
gmcp2  CACAGAGAGC AAGCCACACT CCCGCCCCTA CATGGCC... ..........
smcp2  CACAGAGAGC AAGCCACACT CCCGCCCCTA CATGGCCTAC CTGGAAATTG
gmcp1  CCATGAGGCC AAGCCTCACT CTCGTCCCTA CATGGCGTTT CTTCGGTTCA
smcp1  CCATGAGGCC AAGCCTCACT CTCGTCCCTA CATGGCATTT CTTCAGTTCA
       151                                                 200
gmcp2  .......... .......... ......TGTG GTGGTTTCCT GATAAGAAGG
smcp2  TCACCTCGCA GGGGAAGCAG GTGGCTTGTG GTGGTTTCCT GATAAGAAGG
gmcp1  AGATTTC... AGGGAAGTCT TACAGATGTG GGGGTTTCCT TGTGCGTGAG
smcp1  AGATTTC... AGGGAAATCT TACATATGTG GGGGCTTCCT TGTGCGTGAG
       201                                                 250
gmcp2  GACTTTGTGC TGACGGCTGC GCACTGTGCA GGAAGGTCTG TAACAGTCAC
smcp2  GACTTTGTGC TGACGGCTGC GCACTGTGCA GGAAGGTCTG TAACAGTCAC
gmcp1  GACTTTGTGC TGACAGCAGC TCACTGCCTG GGAAGCTCAG TCAATGTCAT
smcp1  GACTTCGTGC TGACAGCAGC TCACTGCCTG GGAAGCTCAA TTAATGTCAC
       251                                                 300
gmcp2  CCTCGGAGCC CATAACATAC AAAAGAAAGA AGACACATGG CAGAGGCTTG
smcp2  CCTCGGAGCC CATAACATAC AAAAGAAAGA AGACACGTGG CAGAGGCTTG
gmcp1  CCTGGGGGCC CACAACATCA CGGACCGAGA GAGGACCCAG CAGGTCATCC
smcp1  TCTGGGGGCC CACACCATCA CGGACCAAGA GAGGACCCAG CAGGTCATCC
       301                                                 350
GMCP2  AGGTCATAAA ACAGTTTCCT TACCCAAAAT ATGAGCCTG. ..CTGGTCTC
smcp2  AGGTCATAAA ACAGTTTCCT TACCCAAAAT ATGAGCCTG. ..TTGGTGTC
gmcp1  CAGTGAGAAG AGCCATCCCT CACCCACACT ATAATGATGA GACTGGGACC
smcp1  AAGTGAGAAG AGCCATCCCC CACCCAGACT ATAATGATGA AACTTGTGCC
       351                                                 400
gmcp2  CACGACATCA TGTTACTGAA GTTGGAGGAG AAAGCCAACC TGACCCTGGC
smcp2  CACGACATCA TGTTACTGAA GTTGAAGGAG AAAGCCAACC TGACCCTGGC
gmcp1  AATGACATCA TGTTACTGCA GCTGACTAGG AAGGCTGAGA TGACGGATGC
smcp1  AACGACATCA TGTTACTACA GCTGACTAGG AAGGCTGAGA TGACGGATGC
       401                                                 450
gmcp2  CGTGGGGACA CTTCCCCTTC CACCCCATGT CACCTTCATC CGTCCCGGGA
smcp2  CGTGGGGACA CTCCCCCTTC CACCCCATGT CACCTTCATC CGTCCCGGGA
gmcp1  AGTGAGCCTC ATCAATCTGC CCAGGAGCTT GGAGAAGGTG AAGCCAGGGA
smcp1  AGTGAGCCTC ATCAATCTTC CCAGAAGCTT GGAGAAGGTG AAACCAGGGA
       451                                                 500
gmcp2  GAATGTGCCA GGTGGCTGGC TGGGGAAGAA CAGGTGTGAA GGAACCAGCC
smcp2  GAATGTGCCA GGTGGCTGGC TGGGGAAGGA CAGGTGTGAA GGAACCAGCC
gmcp1  TGATGTGCAG TGTGGCTGGC TGGGGGCAAC TGGGGGTAAA TATGCCCTCT
smcp1  TGATGTGCAG TGTGGCTGGC TGGGGGCAAC TGGGGGTAAA TATGCCCTCT
       501                                                 550
gmcp2  TCCAGCACTC TGCAAGAGGT GAAGCTGAGA CTC...ATGG AGCCCCGGGC
smcp2  TCCAGCACTC TGCAAGAGGT GAAGCTGAGA CTC...ATGG AGCCCCGGGC
gmcp1  GCAGACAAAC TACAGGAGGT GGATCTTGAA GTCCAAAGTG AGGAGAAATG
smcp1  GCAGACAAAC TACAGGAGGT GGATCTTGAA GTCCAAAGAG AGGAGAAATG
```

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        551                                                     600
gmcp2   CTGTAGCCAC TTCCCTGCTT TTGACCACAA TCTCCAGCTG TGTGTGGGCA
smcp2   CTGTCGCCAC TTCCGTGCTT TTGACCACAA TCTCCAGCTG TGTGTAGGCA
gmcp1   TATTGCTCGC TTCAAAGACT ACATCCCCAT CACGCAGATA TGTGCTGGAG
smcp1   TATTGCTCGC TTCAAAGACT ACATCCCCGT CACGCAGATA TGTGCTGGAG
        601                                                     650
gmcp2   ATCCCCAGAG CACAAAATCT GCTTTTAAGG GAGACTCAGG GGGCCCTCTT
smcp2   ATCCCCAGAG CACAAAATCT GCATTTAAGG GAGACTCAGG GGGCCCTCTT
gmcp1   ATCCAAGCAA AAGGAAGGAT TCTTTCTTGG GAGACTCTGG GGGCCCTCTT
smcp1   ATCCAAGCAA AAGGAAAGAT TCTTTCTTGG GTGACTCTGG GGGCCCACTT
        651                                                     700
gmcp2   CTGTGTGCTG GGGTGGCCCA AGGCATTGTC TCCTATGGAC TGTTCAGTGC
smcp2   CTGTGTGCTG GGGTGGCCCA GGGCATTGTC TCCTATGGAC TGTCCAGTGC
gmcp1   C.......... .......... .......... .......... ..........
smcp1   GTGTGTGATG GTGTGGCCCA GGGCATTGTG TCCTATGGAA AAGATGATGG
        701                                                     750
gmcp2   AAAGCCCCCC GCCGTCTTCA CCCGGATCTC CCCTTACCGG CCCTGGATCG
smcp2   AAAGCCCCCT GCTGTCTTCA CCCGGATCTC CCCTTACCGG CCCTGGATCG
gmcp1   .......... .......... .......... .......... ..........
smcp1   GACAACTCCA AATGTCTACA CCAGAATCTC CAGCTTTCTG TCCTGGATCC
        751                                                     800
gmcp2   ATGAAGTCCT GAAAGAAAAT TAA..... .......... ..........
smcp2   ATGAGGTCCT GAAAGAGAAT TAACCTGGAA CCTGGGCCAG CCTGAGGAGA
gmcp1   .......... .......... .......... .......... ..........
smcp1   AGAGAACAAT GAGACAGTAC AAAAACCAGG GGATCAGCAT AATGTGTCCT
        801                                                     850
gmcp2   .......... .......... .......... .......... ..........
smcp2   AACCAGAGCA GGACTCCGGC AGGTTCTCGG TGCCACTCAC CCTGGATCTG
gmcp1   .......... .......... .......... .......... ..........
smcp1   TGAGATGGAC CCCTCCATCT TCCCTGGGAT TGGAAGCATT GGTCAAAGTG
        851                                                     900
gmcp2   .......... .......... .......... .......... ..........
smcp2   CCTCTGGTTT TCCTCTTAAA CCCCCGTCAC GTCCCTAATC CTCAGGAAGG
gmcp1   .......... .......... .......... .......... ..........
smcp1   TGTGGAGGAA GGGTGCCTGG AACTTAATAA ACATTCATCT CTTGAAAAGT
        901                                                     948
gmcp
2  .......... .......... .......... .......... ........
smcp2   GTGGTTCAGA TCATAGAATT CCCAATAAAT TTCAGTGAAC ACCCGGCA
gmcp1   .......... .......... .......... .......... ........
smcp1   .......... .......... .......... .......... ........
```

Fig. 5.11 Comparison of currently obtained cDNA nucleotide sequences for GMCP II (full length enzyme clone including the signal sequence and the 39 bp deletion), SMCP II (full length enzyme clone including the signal sequence as well as 5' and 3' non-coding regions), GMCP I (P26 to P2 region) and SMCP I (full length enzyme clone including 3' non-coding region.). The positions of the start codons (ATG) for GMCP II and SMCP II, the codons for the IIGG residues at the start of the mature enzyme peptide sequences and the stop codons (TAA and TGA) for GMCP II, SMCP II and SMCP I are all shown in bold type. See text for comparative sequence identities.

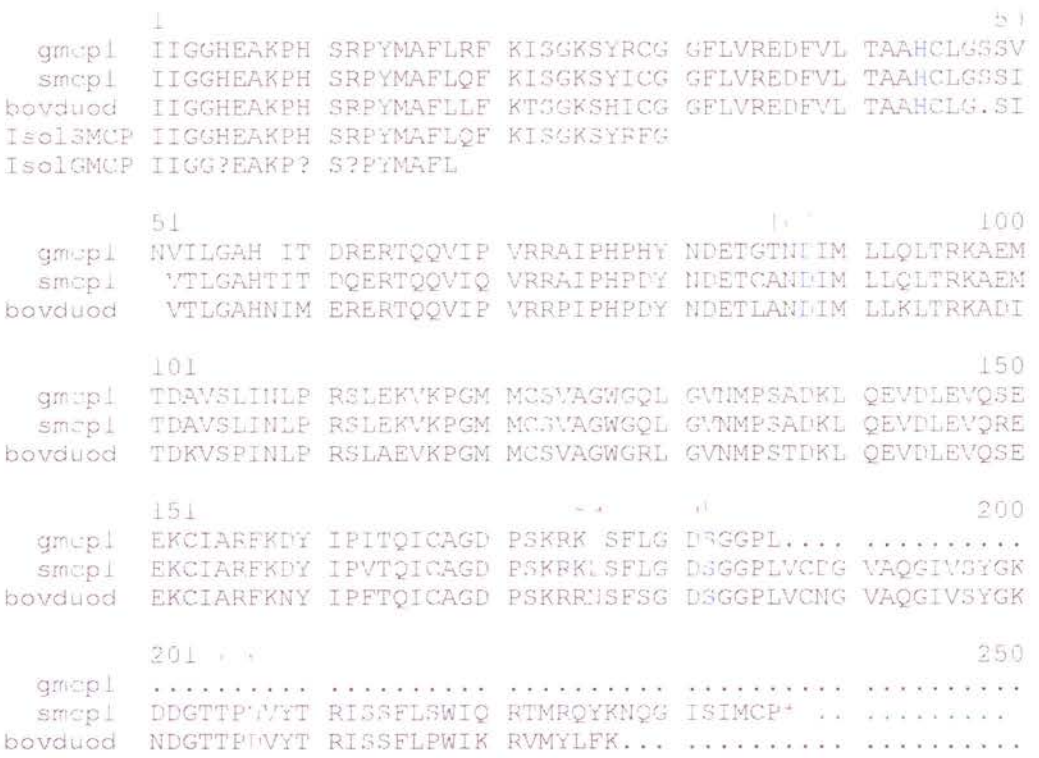


Fig. 5.12 Deduced amino acid sequences from the SMCP 1 (smcp1) and GMCP 1 (gmcp1) cDNA sequences compared with NH_2-terminal amino acid sequences obtained from isolated SMCP (Isol SMCP) and GMCP (Isol GMCP) as well as the whole amino acid sequence for isolated bovine duodenase (Bov Duod) (all shown in standard single letter notation). Note the high degree of identity between the deduced amino acid sequences for SMCP and GMCP as well as the isolated proteases and bovine duodenase ? = undetermined residue; * = stop codon site.; green residues – potential glycosylation sites. The catalytic triad of His, Asp and Ser residues are shown in blue whilst the S_1 active site residues are shown in red. The standard chymotrypsinogen numbered positions of these residues are shown above in red.

5.5 Discussion

This Chapter describes the successful cloning and sequencing of cDNAs encoding goat mast cell proteases. Cloned central fragments for GMCP II were initially isolated from total RNA preparations prepared from day 7 BMMC cell pellets grown in rOvIL-3. This time point in the culture corresponded with increasing GMCP expression in these cells *in vitro* (Chapter 7).

After separating the PCR products by gel electrophoresis, it soon became apparent that the resulting PCR products were too large to be amplified cDNA for GMCP II. Also, sequence analysis of the products demonstrated the presence of two non-coding regions within the sequence, which corresponded to the positions of two introns present in genomic clones isolated previously from ovine BMMC. These two findings suggested that the GMCP II PCR products had been amplified from either unprocessed mRNA, in which intron-containing sequences had not been completely spliced out, or from contaminating genomic DNA. Since the aim of this study was to isolate cDNA coding for GMCP and not to define the enzyme's genomic DNA structure, steps were taken to isolate the fully processed mRNA component from the total RNA samples. As yet, therefore, little further is known about the number or positions of introns within the genes encoding GMCP, although recent data obtained from ovine genomic sequences suggests that there may be as many as four introns and five exons in the equivalent SMCP II gene (S. Macaleese, personal communication).

The message selection procedures for isolating and transcribing mRNA into cDNA proved successful, allowing the cloning and sequencing of 3 overlapping GMCP cDNA fragments which, when concatenated, covered an area from 22bp before the start of the coding sequence for the mature protein through to the 3' poly dA tail of the mRNA. This contiguous assembly of fragments was highly homologous with an equivalent SMCP II cDNA previously isolated from ovine BMMC, indicating that cells from both species produced enzymes which were potentially very similar in terms of their respective amino acid sequences.

This was confirmed when a clone containing almost the whole coding region for the GMCP II pro-peptide was amplified using primers that bound to an area coding for the SMCP II signal peptide and to an area incorporating the 3' stop codon. This 'full length' clone was almost identical to the contiguous GMCP fragment clones with the exception of two areas. In the first place, the full length clone's 5' signal

peptide sequence was markedly different to the signal sequence obtained from the original 5' GMCP cDNA fragment (Fig. 5.7). This indicated that the ovine P19 primer used to amplify the full length clone was preferentially binding to a different signal peptide sequence to that present in the original oligo dT amplified 5' GMCP cDNA fragment. This alternative signal peptide has been previously amplified from 5' polyA tailed sheep mRNA samples (S. Macaleese, personal communication) and suggests the presence of two different signal peptides encoding similar proteins in both species. It is possible therefore, that the GMCP gene is capable of alternatively splicing 5' exons containing different signal peptides onto the rest of the GMCP II mRNA transcript. The presence of GMCP mRNA transcripts encoding proteins with different signal peptide sequences may indicate that different forms of translated GMCP II could be stored in different ways within the cell, although the functional significance of this remains to be determined. The second area of difference between the contiguous GMCP II sequence and the full length clone lay between base pairs 137 and 177 where there is an apparent gap in the the full length clone's nucleotide sequence (Fig. 5.7). The reasons behind this deletion are also unknown, but it may have occurred during the first cycle of the initial PCR amplification when incomplete strand separation of the RT product could have produced a 'looped out' area of cDNA that was by-passed by the Taq DNA polymerase. Alternatively, it may also indicate the presence of yet another gene product due to the splicing of an alternative 5' exon sequence containing the deletion. However, to prove this, sequencing of at least three different clones from independent PCR products amplified using the original primers would be required to rule out the possibility of errors during the PCR reaction. Overall however, this means that although this particular clone was subsequently used successfully as a probe (Fig.5.10 a and b), it may not be suitable for use in a protein expression system since it would possibly code for a defective protein product.

Despite these anomalies, the cDNA sequence coding for the GMCP II pro-peptide in the whole enzyme clone still showed a high degree of identity with the GMCP II fragment clones and the BMMC derived clone for SMCP II. This finding further confirmed the similarities between the goat and sheep enzymes at the DNA level. Peptide translations were carried out on the two GMCP II sequences and the SMCP II sequence to check their similarity in terms of their deduced amino acid sequences (Fig. 5.8). Apart from the different signal peptide obtained from the full length GMCP II cDNA clone, all three showed a high degree of identity with only 5 out of 229 residues difference between the GMCP II and SMCP II mature enzyme deduced amino acid sequences. Both also contained the G-E dipeptide found attached to the immature pro-enzyme form of the protein as it emerges from the rough endoplasmic reticulum following translation. This dipeptide is removed prior to packaging within the mast cell granule, allowing the enzymes to alter their tertiary structure by folding to produce the optimal active site conformation for substrate cleavage (Urata *et al.*, 1993). Immediately after the pro-enzyme di-peptide, the mature enzyme sequences from both species started with an I-I-G-G tetrapeptide, which is common to the majority of mast cell chymase enzymes sequenced so far (Springman and Serafin, 1995).

Also common to both enzymes are the histidine$_{57}$, aspartate$_{102}$ and serine-$_{195}$ residues (chymotrypsinogen numbering), that make up the 'catalytic triad' which is associated with all known serine esterases (Springman and Serafin, 1995). However, somewhat unexpectedly given the biochemical characteristics of GMCP (Chapter 3), sequence position 226 did not contain either aspartate or glutamate residues. These negatively charged residues, when present in this position (or possibly also at position 189, see below) divide the base of the S_1 portion of the substrate binding pocket to produce an active site conformation that is associated with the dual chymase and tryptase specificity of bovine duodenase and human cathepsin

G. (Zamolodchikova *et al.*, 1995b; Hof *et al.*, 1996). Instead, both enzymes contained an alanine$_{226}$ and a serine$_{189}$, which has previously been associated with a chymotrypsin-like substrate specificity in the rat intestinal chymase, RMCP II (Remington, Woodbury, Ross, Reynolds, Matthews and Neurath, 1988).

It was also noted that the deduced amino acid sequences for these enzymes exhibited poor identity with the NH_2-terminal amino acid sequences previously obtained for isolated SMCP and GMCP (Pemberton *et al.*, 1997a; Chapter 3), as well as the complete amino acid sequence for bovine duodenase (Zamolodchikova *et al.* 1995b), which appears to be the bovine equivalent of GMCP and SMCP (Pemberton *et al.* 1997a). In contrast, a search carried out on the EMBL database (accessed via Seqnet, see Chapter 2.14.2) showed that both enzymes had a greater DNA sequence identity (84 to 86 %) with a human heart chymase enzyme which acts as an important angiotensin I converting enzyme within cardiac muscle (Urata, Kinoshita, Perez, Misono, Bumpus, Graham and Husain, 1991). Overall, the findings indicated that these cDNAs clones were coding for novel mast cell chymases that are present in both species, but have yet to be isolated or detected as discrete proteins either *in vivo* or *in vitro*. As outlined above, the enzymes were classified as SMCP II and GMCP II, and the information gained from their cDNA sequences was used to construct more-specific primers for amplifying cDNA for GMCP I and SMCP I.

The GMCP I NH_2-terminus specific primer, in conjunction with a primer specific for an area of consensus between the amino acid sequences obtained for GMCP II and bovine duodenase (P2), was successful in producing a positive cDNA clone after only one amplification step. This may reflect on the relative amounts of cDNA coding for GMCP II (which always required two amplification steps) compared with cDNA for GMCP I in the RT samples, although no quantitative studies were carried out to investigate this further. The cDNA clone for GMCP I coded for about two thirds of the enzyme with the 3' end currently left unsequenced

due to time constraints within the project. However, this 3' sequencing has been recently carried out for SMCP I (S.Macaleese, personal communication, Fig. 5.11) producing a clone which enables the entire mature enzyme nominal peptide sequence to be deduced (Fig. 5.12). The deduced amino acid sequence for GMCP I demonstrates a high degree of homology with the SMCP I sequence and also shows a much higher identity with the NH_2-terminal sequences for isolated SMCP I, GMCP I and bovine duodenase (Fig. 5.12). This is especially noTable for residues 5,7 and 19 which contain histidine, alanine and phenylalanine respectively instead of the threonine, serine and tyrosine residues found in the GMCP II N-terminus (Fig. 5.8). Indeed, the sequence identity of 83.3% for the GMCP I clone and the NH_2-terminus of isolated GMCP would probably be much higher if it wasn't for the 3 undetermined residues in the isolated enzyme's sequence.

Like GMCP II, the deduced amino acid sequences for GMCP I and SMCP I also contain the His, Asp, Ser catalytic triad associated with serine esterase activity and both enzymes also exhibited potential N-linked glycosylation sites, although their precise locations vary slightly between SMCP I and GMCP I (Fig. 5.12). These glycosylation sites are not always linked to carbohydrates, but in the case of SMCP I the presence of glycosylated and non-glycosylated forms of the enzyme would possibly explain the presence doublet bands found in SDS-PAGE separated samples of purified SMCP and immunoaffinity purified GMCP (A. Pemberton, personal communication, Chapter 3).

Within the active site itself, the residue at position 226 has yet to be determined for GMCP I although SMCP I has an $asparagine_{226}$ residue which in itself, would not suggest the presence of dual specificity. However, SMCP I and GMCP I do possess an $aspartate_{189}$ which, on the basis of structural modelling for human cathepsin G, lies directly opposite residue 226 at the base of the S1 pocket (Hof et. al. 1996). In bovine duodenase, on the other hand, the position of these

residues is reversed, with an asparagine$_{189}$ and an aspartate $_{226}$, which is the conventional arrangement for dual specificity (Zamolodchikova et. al., 1995b). Given the spatial arrangement of residues at the two sites within the substrate binding pocket, it is possible that an aspartate residue at either position may be sufficient to produce the dual activity that is characteristic of GMCP I (Chapter 3) and SMCP I (Pemberton *et al.*, 1997a), although further computer modelling studies based on their complete amino acid sequences would be required to confirm this.

In summary, cDNAs coding for two separate GMCP enzymes have been cloned from goat BMMCs. Comparisons between a 5' and a whole length cDNA clone for GMCP II indicated that alternative splicing may occur during RNA processing. This may result in the production of transcription products that code for different signal peptides. The whole length cDNA for GMCP II encodes an enzyme which, in terms of it's deduced amino acid sequence, is more closely related to a human heart chymase enzyme than to isolated GMCP, isolated SMCP or bovine duodenase. In terms of its potential active site conformation, it also exhibits the structural properties of a chymotrypsin-like enzyme rather than a dual specific enzyme. In contrast, a second cDNA isolated for GMCP I encodes approximately two thirds of an enzyme whose deduced amino acid sequence closely resembles isolated GMCP, isolated SMCP and bovine duodenase. Although the the complete sequence for its active site residues has not yet been determined, the presence of an aspartate$_{189}$ residue indicates a possible structural characteristic by which dual substrate specificity may occur.

CHAPTER 6

ABOMASAL NEMATODE INFECTIONS IN GOATS: A COMPARISON OF MAST CELL, EOSINOPHIL AND TOTAL SERUM IgE RESPONSES IN YEARLINGS, KIDS AND LAMBS UNDERGOING PRIMARY AND SECONDARY CHALLENGE INFECTIONS WITH *T. circumcincta*

6.1 Introduction

Recent studies (Huntley *et al.*, 1995), analysed the role of cellular immune responses in previously infected adult goats and sheep exposed to an experimental mixed challenge of *T. circumcincta* and *T. vitrinus* L_3. As in earlier studies (LeJambre and Royal, 1976; LeJambre, 1984; reviewed Lloyd, 1987; McKenna, 1984; Pomroy *et al.*, 1986), the findings demonstrated that adult does, despite previous exposure to nematodes in the field, were generally incapable of restricting larval establishment when compared with equivalently challenged adult ewes. Fundamental differences were also observed in the MMC and GL numbers as well as SMCP concentrations in goat intestinal tissues compared to sheep tissues (see general introduction).

The aim of this chapter is to extend these preliminary findings by examining cellular and humoral immune responses in groups of yearling goats, kids and lambs undergoing both primary and secondary challenge infections with the abomasal parasite *T. circumcincta.* This was carried out with emphasis on examining differences in the MMC and GL responses shown by the two species. In contrast to the previous study, where the adult animals were maintained on pasture before being treated with anthelmintic and exposed to an experimental challenge, the animals in this study were all kept under worm-free conditions prior to the start of the experiment. This allowed assessments to be made on comparative primary responses to infection shown by lambs and goats as well as providing novel data on both primary and secondary responses shown by goat yearlings and goat kids. The use of a controlled experimental trickle-challenge regime for 'priming' the continuously challenged animals enabled comparisons to be made on the responses of animals that have undergone exposure to known numbers of L_3 prior to a secondary challenge infection. The experimental challenge of worm-free animals also circumvented potential complications associated with the frequent use of anthelmintics in the

naturally infected animals used in earlier experiments. This has previously been shown to affect the development of acquired immunity by limiting host exposure to parasites in the field prior to experimental challenge (Gibson *et al.* 1970; Barger, 1988).

The use of yearlings and kids also allowed assessments to be made as to whether an age-related immune responsiveness to nematodes occurs in goats in a similar manner to that observed previously in sheep (Colditz *et al.* 1996). In addition, the raising of polyclonal antibodies to GMCP and it's cross reactivity with SMCP (Chapter 3) enabled mast cell responses to be determined, using GMCP-specific reagents for immunohistological and tissue ELISA analyses. This was in contrast to the previous study (Huntley *et al.*, 1995), where only reagents specific for SMCP were available. Finally, the availability of a monoclonal antibody that recognises both caprine and ovine IgE (Kooyman *et al.*, 1997) allowed the first comparative measurements for total serum IgE levels in response nematode infections, in these two species. Elevated levels of this immunoglobulin isotype have been described in a wide variety of parasitic infections (Jarrett and Miller, 1982) and have also been implicated in mast cell activation (reviewed Sutton and Gould, 1993).

6.2 Materials and methods

6.2.1 Animals

Eight eighteen month old Scottish cashmere goats (yearlings), ten six month old Scottish Blackface cross lambs (lambs) and ten six month old feral cross kids (kids) were used in the experiment. The animals were all born and reared indoors at the MRI's Firth Mains Farm, Penicuik, Mid-Lothian. They had access to a crepe feed (Dalgettys Lamb ration) and hay. At ten weeks of age they were weaned and offered a daily ration of concentrate (Moredun Sheep Nuts, Dalgettys) and hay *ad-lib.*

At the start of the experiment, animals were randomly allocated into one of two groups. The first group contained five yearlings, five kids and five lambs, the

second group contained three yearlings, five kids and five lambs. The second group, termed the continuously infected (CI) group, commenced a primary trickle-challenge regime whilst the first group, termed the challenge control (CC) group, continued to be maintained under worm free conditions.

6.2.2 Challenge protocol

Table 6.1 outlines the experimental timetable used during the challenge period for groups of animals undergoing both primary and secondary infection regimes. The CI group commenced their primary trickle-challenge at day 0. This consisted of 2000 *T. circumcincta* L_3 given per five days a week *per os* for 6 weeks, followed by a single 50, 000 *T. circumcincta* L_3 challenge dose on day 49. The CC group were given the single 50, 000 *T. circumcincta* L_3 challenge dose at day 49.

All animals were bled by jugular venipuncture at weekly intervals to provide serum and EDTA treated whole blood for IgE and peripheral eosinophil counts respectively (see below). Rectal faecal samples were also taken at weekly intervals from CI animals to monitor their faecal egg output throughout the trickle challenge period. Seven days after the administration of the *T. circumcincta* 50 000L_3 challenge, all animals were euthanased and the intestinal tissues processed as described in 2.3 and below.

Group No.	Animals (n)	Treatment	No. doses	Challenge	Challenge day	Kill day
1	CC yearlings (3)	-	-	50,000 L_3	49	56
2	CI yearlings (5)	2000 L_3 five times per week	35	50,000 L_3	49	56
1	CC kids (5)	-	-	50,000 L_3	49	56
2	CI kids (5)	2000 L_3 five times per week	35	50,000 L_3	49	56
1	CC lambs (5)	-	-	50,000 L_3	49	56
2	CI lambs (5)	2000 L_3 five times per week	35	50,000 L_3	49	56

Table 6.1 Outline of the experimental protocol for challenging the continuously infected (CI) and challenge control (CC) groups of animals. L_3 = *T. circumcincta* infective third stage larvae.

6.2.3 Faecal egg counts

These were kindly performed by members of Dr. F. Jackson's laboratory using a modified flotation technique as described by Christie and Jackson (1982). Briefly, weighed faecal samples were emulsified in 10mls of tap water per gram of faeces. A 10ml sub-sample was removed and passed over a 1mm aperture sieve. The retentate was washed with an additional 5ml of tap water, compressed to recover as much fluid as possible, and then discarded. The filtrate was centrifuged at 1000 rpm (228 x g) for 2 minutes. The resulting pellet was re-suspended in 12ml saturated NaCl prior to centrifugation at 1000rpm for another 2 minutes. The upper 1 to 2 mls of supernatant was collected and transferred into a 4ml disposable polystyrene cuvette (LIP Ltd., Shipley). The cuvette was filled with saturated NaCl solution and sealed. Counts were carried out on a compound microscope using a calibrated eyepiece graticule (Miller square) at x 40 magnification. Two longitudinal traverses of the cuvette were performed, and the numbers of eggs falling inside the boundaries of the large graticule square multiplied by 3 or those within the small square by 9 to obtain the total numbers of eggs per gram of faeces.

6.2.4 Peripheral blood eosinophil counts

These were kindly performed by J. Lightbody of the Biochemistry Division at Moredun Research Institute. A 50µl sample of fresh EDTA treated whole blood was added to 450µl of Carpentier's eosinophil staining solution (Appendix A) and the eosinophils counted using a "Fast-Read 10" disposable counting chamber (Immune Systems Ltd., Bristol, U.K.). Blood eosinophil numbers are expressed as cells x 10^9 per litre of whole blood.

6.2.5 Post mortem processing of tissues

After euthanasia, the entire gastro-intestinal tract was removed and the proximal end of the duodenum located. The small intestine was separated from the abomasum and a section of jejunum taken for GMCP ELISA analysis as outlined in 2.3 The abomasum was opened along it's greater curvature and the contents collected, individual abomasal pH was measured immediately using a stick pH meter (Whatman pH µ-Sensor). An abomasal fold was taken for GMCP ELISA analysis, whilst an additional fold was fixed in 4% paraformaldehyde/PBS as described in 2.8. The abomasum and it's contents were then soaked in a 0.85% saline solution at 37 ^{o}C for 4 hours (Jackson, Jackson and Smith, 1984). Afterthis saline incubation, the superficial mucosa was removed from the remainder of the abomasum , which was then discarded. The contents, including the mucosa, were made up to 5 litres with 0.85% saline , and a 500ml (10%) subsample taken and fixed with 20ml formalin.

6.2.6 Abomasal worm counts

A 100ml aliquot (representing 2% of the total worm population), was taken from each of the fixed subsamples recovered from the abomasum and stained with 10-15mls of helminthological iodine (Appendix A). The stained sample was examined under a stereo-microscope and the *T. circumcincta* larvae counted and staged as

early, mid or late L_4 or as male or female L_5/early adults as described by Denham (1969).

6.2.7 Histochemical and immunohistochemical evaluation of tissue sections for MMC, GL and eosinophils.

These were carried out on 4% paraformaldehyde fixed tissues as described in 2.8. Immunohistochemical staining was carried out using rabbit polyclonal antibody to GMCP. Final results were expressed as cells/0.2mm^2 of abomasal tissue.

6.2.8 ELISAs of abomasal and jejunal tissues for GMCP/SMCP

These were carried out as described in Chapter 2.12 using a double antibody sandwich ELISA for GMCP. Final results were quoted as μg GMCP/SMCP per gram of wet weight tissue.

6.2.9 Serum total IgE assays

These were carried out in duplicate as outlined in Chapter 2.13 on reduced serum samples. The results were expressed as μg of total IgE/ml of serum.

6.3 Results

6.3.1 Faecal egg counts from CI animals

The mean (± SD) weekly faecal egg counts of the CI yearlings, kids and lambs undergoing the trickle challenge infection regime are shown in Fig. 6.1. (faecal egg counts from worm-free CC animals were all negative prior to the single challenge at day 49). At day 21, the lambs produced significantly more eggs than the yearlings and kids whilst, at day 35, both the yearlings and the lambs produced significantly more eggs than the kids ($p < 0.05$). Between days 42 and 49, the lambs produced significantly more eggs than the yearlings ($p < 0.05$). However, after day 49 there were no significant differences in the egg outputs of the yearlings, kids or lambs.

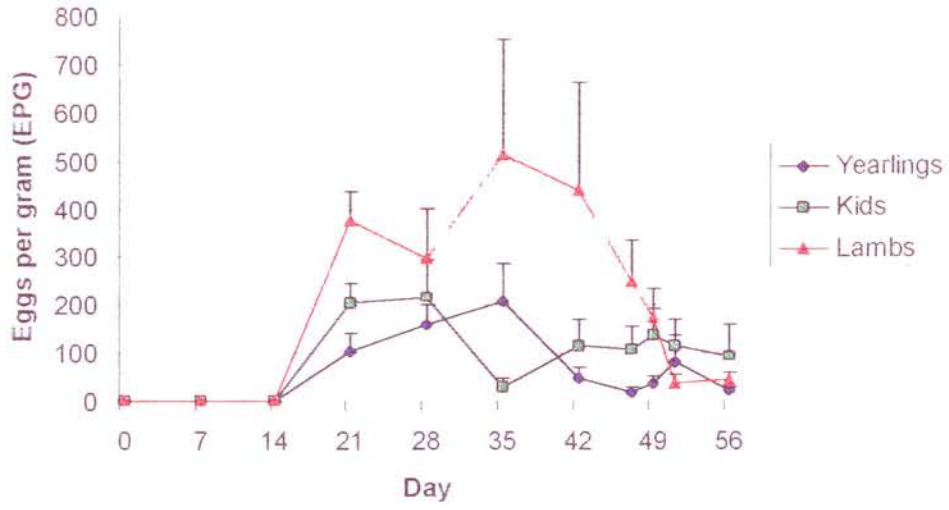


Fig. 6.1 Mean (± sem) numbers of eggs per gram of faeces recovered from CI animals undergoing primary trickle challenge with 2000 *T. circumcincta* L_3 5 times weekly.

6.3.2 Abomasal worm burdens

Table 6.2 shows the summary mean (± SD), abomasal pHs, L_4 stages, L_5/adult and total worm numbers as well as the percentage establishment of larvae in the CI and CC yearlings, kids and lambs. Figure 6.2a shows the relative numbers of early, mid and late L_4 stage larvae recovered from individuals in the CC group whilst Figure 6.2b shows the relative numbers of the combined L_4 stages and L_5/adults recovered from individuals in the CI group.

Both the CC and CI yearlings and kids had significantly higher abomasal pHs than the equivalent CC and CI lambs, whilst the CI kids also had significantly higher abomasal pHs than the CC kids ($P < 0.05$). In the CC group, the mean total larval burdens and mean percentage of larvae established were marginally higher in the kids than either the yearlings or lambs, although the differences were not significant ($P > 0.05$). It was also found that the kids contained significantly more mid L_4 stage larvae than the yearlings and lambs ($P < 0.001$), where the majority of larvae had

continued to develop to the late L_4 stage (Table 6.2 and Fig. 6.2a). Due to the short interval (seven days) between challenge and post mortem, none of the worms in the CC animals had developed through to the L_5/adult stages.

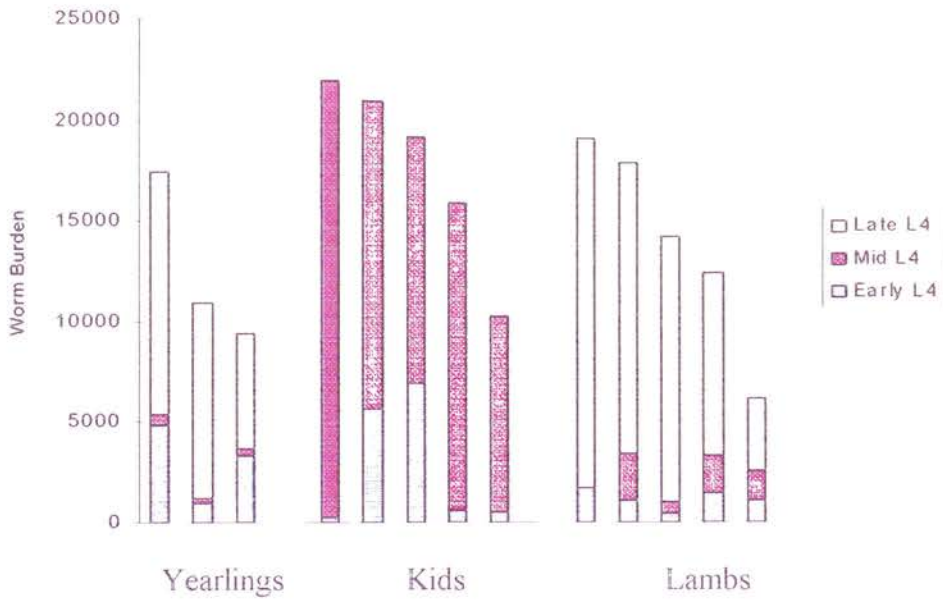
In the CI group the kids harboured significantly higher total numbers of worms than either the CI lambs or the CC kids ($P < 0.05$), although neither the CI yearlings or lambs contained significantly higher total worm numbers than the equivalent CC animals. The mean total worm burdens present in the CI yearlings were also considerably higher than the mean total worm burdens present in the CI lambs. However,as can be seen in Fig. 6.2b the difference was not significant due to considerable variations in the total numbers of worms recovered from the individual CI yearlings.

Percentage larval establishment figures showed that only approximately half the numbers of larvae established in the CI lambs throughout the experiment when compared to the CI yearlings and kids. Establishment ratios calculated for the two groups (CC: CI) demonstrated a marked decrease in the CI lambs (1: 0.45) when compared to either the CI yearlings (1: 0.81) or the CI kids (1: 0.69).

Animal (n)	Abomasal pH	Early L_4	Mid L_4	Late L_4	L_5/Adult	Total	% Estd.
CC							
Yearlings (3)	4.13 (± 0.29)[d]	3073 (± 1133)	341 (±93)	9138 (± 1841)	0	12551 (± 2466)	25.1
Kids (5)	4.06 (± 0.14)[c]	2778 (± 1437)	14834 (± 2015)[b,c]	0	0	17611 (± 2118)	35.2
Lambs (5)	2.36 (± 0.4)	1158 (± 219)	1242 (± 423)	11499 (± 2388)	0	13900 (± 2278)	27.8
CI							
Yearlings (5)	5.06 (± 0.39)[d]	14343 (± 5264)	0	2123 (± 1109)[a]	7899 (± 1266)	24361 (± 5354)	20.3
Kids (5)	5.66 (± 0.49)[a,c]	12583 (± 3534)[a]	0	1106 (± 793)	15371 (± 3684)	29060 (± 1278)[a,e]	24.2
Lambs (5)	3.6 (± 0.47)	4315 (± 1546)[a]	0	0	10644 (± 2207)	14969 (± 2679)	12.5

Table 6.2 Mean (± SD) abomasal pHs, post mortem worm burdens and % establishment of total larvae administered (% Estd.) results from the CI and CC yearlings, kids and lambs. Superscripts denote significance ($p< 0.05$). a = CI versus CC infected animals, b = between CC kids and yearlings, c = between CC or CI kids and lambs d = between CC or CI yearlings and lambs and e = between CI kids and lambs (see also text). Results for individual animals are in Appendix B (pp i & ii).

a



b

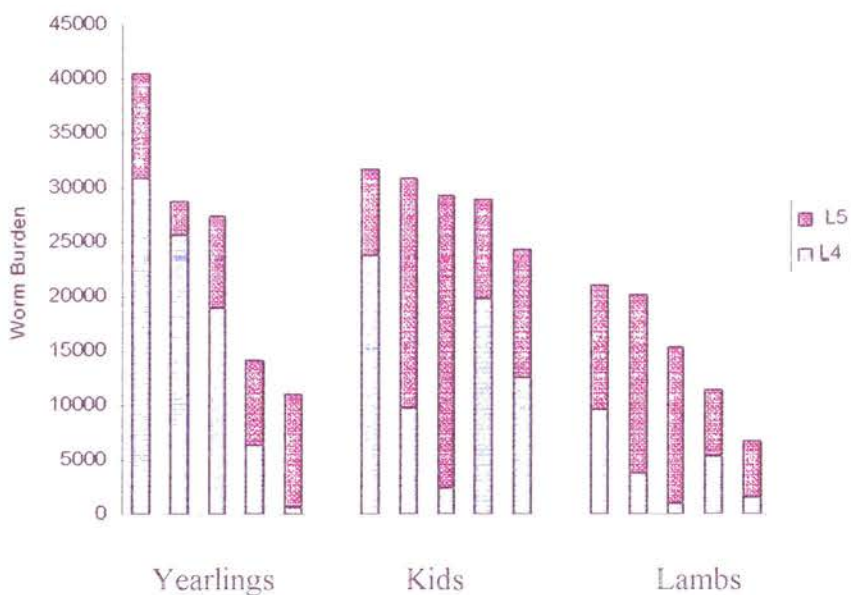


Fig. 6.2. a) Numbers of early, mid and late stage L_4 larvae recovered from CC animals seven days after a single primary challenge infection with 50, 000 *T.circumcincta* L_3 . b) Numbers of combined L_4 stage larvae and L5/adult worms recovered from CI animals seven days after undergoing a secondary challenge infection with 50, 000 *T.circumcincta* L_3.

Animal (n)	Toluidine blue (MMC)	Carbol chromotrope (GL)	% GL	α-GMCP	Eosinophils
CC					
Yearlings (3)	4.5 (± 2.9)	4.7 (± 1.0)[b,c]	51[b,c]	5.9 (± 3.1)	3.1 (± 2.4)
Kids (5)	3.1 (± 2.0)	0.12 (± 0.03)	3.7	6.0 (±2.67)	0.96 (± 0.6)
Lambs (5)	3.6 (± 1.5)	0.32 (± 0.4)	8.1	4.6 (± 2.6)	4.6 (± 2.6)
CI					
Yearlings (5)	12.3 (± 3.7)[a]	19.6 (±6.1)[a,c]	61[c]	12.6 (± 4.0)	5.8 (± 3.7)
Kids (5)	10.3 (± 3.1)[a]	9.2 (± 4.1)[a,d]	47[a,d]	11.1 (± 4.4)	13.3 (± 5.4)[a,d]
Lambs (5)	10.5 (± 3.1)[a]	1.1 (± 1.1)	9.4	10.4 (± 3.5)	3.12 (± 1.5)

Table 6.3 Mean (± SD) abomasal mucosal mast cells (MMC), globule leukocytes (GL), % of total mast cells (combined MMC and GL counts) as GLs (% GL), cells reacting positively with polyclonal antibody to GMCP (α-GMCP) and tissue eosinophil numbers (Eosinophils). All counts represent the number of cells/ 0.2mm^2 section of tissue. Superscripts denote significance ($p < 0.05$). a = CC versus CI animals, b = between CC yearlings and kids, c = between CC or CI yearlings and lambs, d = between CI kids and lambs (see also text). Results for individual animals are in Appendix B (p ii)

6.3.3 Tissue MMC and GL counts.

Table 6.3 shows the mean (± SD) results for MMC and GL counts after staining abomasal tissue sections from both groups with toluidine blue for MMC, carbol chromotrope for GL, and antibody to GMCP (α-GMCP). Toluidine blue stained MMC showed a significant ($P < 0.05$) increase in numbers in the tissues of CI yearlings, kids and lambs, compared to the equivalent CC animals. Mean GL numbers were also increased in the CI compared to CC infected animals, although the increase was significant for the yearlings and kids only ($P < 0.001$). The CC yearlings along with the CI yearlings and kids contained a significantly higher proportion of their total mast cell count (combined MMC and GL numbers) as GL (% GL, table 6.3) than the CC kids and lambs or CI lambs ($P < 0.005$). Tissues stained with antibody to GMCP did not reveal any significant differences in the numbers of positive staining cells

between the CC and CI infected groups, although the mean numbers of positive cells were greater in the CI group.

Correlations between total abomasal worm burdens and the combined cell counts for histochemically stained MMC and GL (total mast cells, see Chapter 3 and Chapter 4) demonstrated inverse relationships in the CC yearlings (R^2= -0.69) and lambs (R^2= -0.53) as well as the CI yearlings (R^2= -0.39) and lambs (R^2= -0.51). Similar analyses on the CC and CI kids demonstrated no relationship and a positive relationship (R^2= 0.40) respectively. Regression analyses also demonstrated that anti-GMCP staining correlated better with the total mast cell counts for the CC group (R^2 = 0.70; $p < 0.0001$) than for the CI group (R^2 = 0.55; $p < 0.002$).

6.3.4 Tissue mast cell protease concentrations

Animal (n)	Abomasum	Jejunum
CC		
Yearling (3)	0.06 (± 0.02)	1.2 (± 0.8)
Kid (5)	0.09 (± 0.02)	1.1 (± 0.7)
Lamb (5)	0.4 (± 0.2)^{b,c}	5.4 (± 3.6)^{b,c}
CI		
Yearling (5)	0.04 (±0.02)	0.3 (± 0.2)
Kid (5)	0.06 (± 0.015)	0.8 (± 0.48)
Lamb (5)	94.1 (± 44.6)^{a,b,c}	18.5 (± 9.0)^{a,b,c}

Table 6.4 Mean (± SD) values for abomasal and jejunal tissue GMCP content. Results expressed as µg/g wet weight tissue. Superscripts denote significance ($P< 0.05$). a = between CC lambs CI lambs, b = between CC or CI lambs and yearlings, c = between CC or CI lambs and kids. Results for individual animals are in Appendix B (p ii).

Table 6.4 shows the mean (±SD) results obtained for the abomasal and jejunal tissue GMCP concentrations for both CC and CI groups. In the CC animals, the jejunal tissues from both species contained more protease than the abomasal tissues, whilst the lamb abomasal and jejunal tissues contained significantly more protease than either the yearling or kid tissues ($p < 0.01$). In the CI animals, there was a highly significant increase in the tissue protease concentrations in the lamb abomasal and jejunal tissues ($P< 0.001$). This was most marked in the abomasal tissues which, in contrast to the

CC lambs, now contained significantly more protease than the CI lamb jejunal tissues ($P < 0.01$). In CI yearlings and kids however, there were slight reductions in the mean tissue protease content for both the abomasal and jejunal tissues when compared with the equivalent CC animals and, although the results were not significant, the jejunal tissues still contained more GMCP than the abomasal tissues.

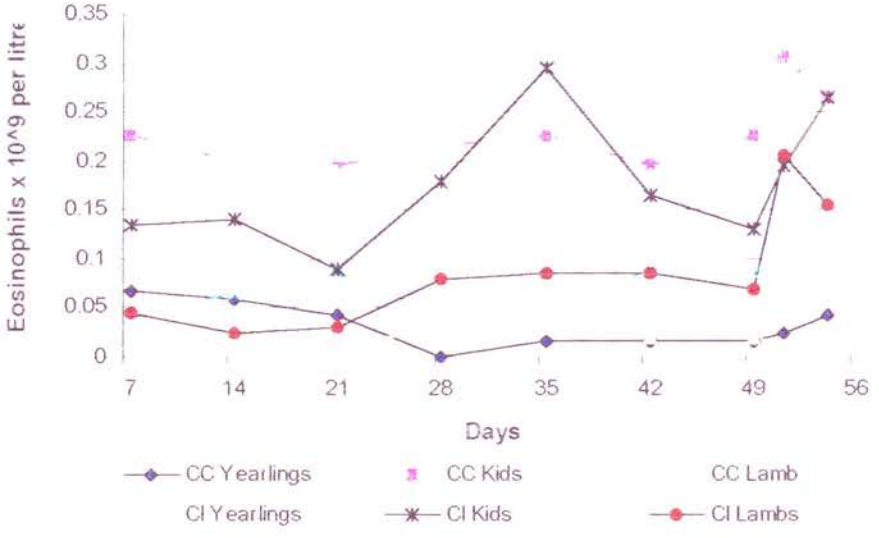


Fig. 6.3 Mean peripheral eosinophil counts obtained from weekly blood samples taken from CC and CI animals (cells x 10^9/litre of whole blood).

6.3.5 Eosinophil counts

Figure 6.3 shows the mean peripheral eosinophil numbers present in weekly blood samples taken throughout the experimental period from yearlings, kids and lambs undergoing both challenge regimes. There were no significant differences in cell numbers noted between the groups at any stage throughout the experimental period. However, the mean values obtained from the CC or CI kids were marginally higher than those obtained from other animals until the 50, 000 *T. circumcincta* L_3 challenge

at day 49. The mean counts obtained from all animals rose in the period between day 49 and day 54, when the last blood sample was taken, although, the increase was not significant ($P > 0.05$) for any group.

Table 6.3 also shows the mean (± SD) results for eosinophil counts in carbol chromotrope stained abomasal tissue sections. The mean values for CI yearlings and kids were both higher than those obtained from equivalent CC animals, although only the kid results were significantly elevated ($P < 0.05$). Conversely, the numbers of eosinophils present in the CI lamb tissues were lower than the CC infected lambs, but the decrease was not significant ($P > 0.05$).

6.3.6 Total serum IgE concentrations

Figure 6.4 shows a typical total serum IgE dot blot of reduced yearling serum probed with the 1E7 monoclonal antibody for caprine and ovine IgE (see 2.13). Figure 6.5 shows the comparative mean (± SD) total serum IgE concentrations for CC and CI yearlings (A), CC and CI infected kids (B) and CC and CI infected lambs (C) obtained using this technique.

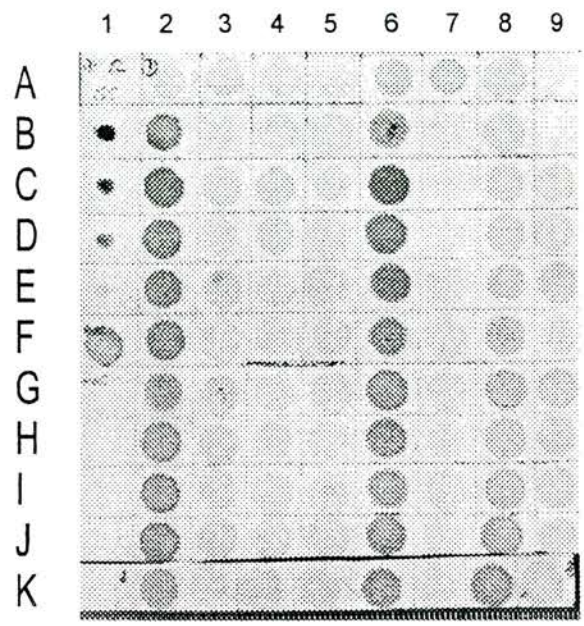


Fig. 6.4 Total serum IgE dot blots of weekly serum samples from CC yearlings (column 2 to column 4) and CI yearlings (column 5 to column 9). Samples from individual animals run from row A (samples taken at the start of the trickle challenge) to row K (samples taken at post mortem). Also shown are the 0.125 to 7 μg/ml recombinant IgE standards (1B to 1E) as well as positive and negative control samples consisting of ADCM challenged doe gastric lymph (1F), specific pathogen free (SPF) lamb serum (1G) and worm-free goat serum (1H).

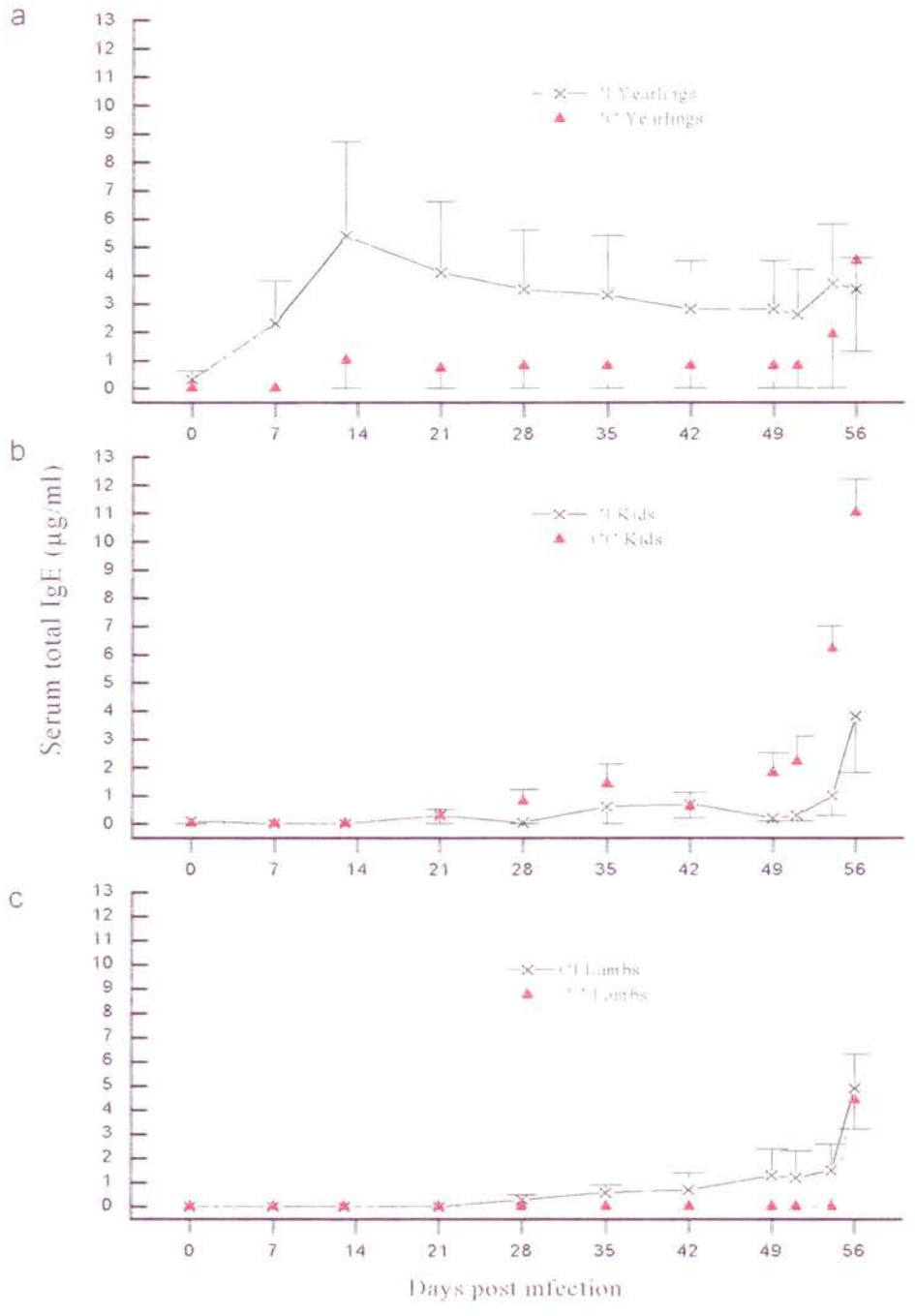


Fig. 6.5 Mean (± SD) total serum IgE concentrations in µg/ml from weekly blood samples taken from CC and CI yearlings (a), kids (b), and lambs (c). CI animals given 2000 *T. circumcincta* L_3 five times a week until day 49; CC animals maintained worm-free until day 49 when both CC and CI animals were challenged with 50,000 *T. circumcincta* L_3.

The CI yearlings demonstrated a strong initial mean total serum IgE response, which was maintained throughout the primary trickle-challenge period (Fig. 6.5a). However, due to a high degree of variability between individuals, the results were not significantly different to the levels obtained from equivalent worm-free CC animals. The CC yearlings also demonstrated a slight rise in serum total IgE levels between day 7 and day 14 despite being maintained under worm-free conditions until day 49 (although this response was mainly due to a single animal; Fig 6.4, column 2). Neither the CC or CI kids demonstrated significant elevations in their mean serum total IgE concentrations throughout the trickle challenge period. After the 50,000 L_3 challenge, however, both groups showed increases by day 54 (Fig. 6.5 b). The greatest responses were found in the CC kids which produced significantly more total IgE during this period than the CI group ($P< 0.05$). In the lambs, increases in mean serum total IgE levels were not demonstrated in the CI animals until day 28 post infection (Fig. 6.5c), whilst CC lambs were negative for serum IgE throughout the trickle-challenge period. After the 50, 000 L_3 challenge, the IgE concentrations in both lamb groups had risen by day 56. This rise was most significant in the CC lambs ($P<0.01$).

6.4 Discussion

These results demonstrate the inability of goat yearlings and kids to reduce their mean total gastro-intestinal nematode burdens when compared with equivalently challenged lambs. They also highlight differences in kinetics of worm establishment, development and expulsion in the two species, which may result from differences in their respective physiological and immunological responses to nematode infections.

A surprising initial finding was the significant reduction in faecal egg output shown by the CI yearlings and kids when compared with the CI lambs during the

trickle challenge period. This appeared to be at odds with previous studies (Lejambre and Royal, 1976, Lejambre, 1984, Pomroy *et al*, 1986, Huntley *et al.* 1995) which demonstrated that infected adult goats usually shed more eggs than equivalently challenged adult sheep. Faecal egg output is, however, only a measure of nematode fecundity, which may be influenced by a variety of immunological, physiological and environmental factors that do not affect larval establishment. Reductions in fecundity can therefore occur despite the presence of substantial intestinal nematode populations (Douch and Morum, 1993). This was apparently the case in the current experiment, since worm burdens recovered from CC and CI kids as well as CI yearlings at post-mortem, were larger than the equivalent lambs. Although there were no significant differences in the faecal egg outputs from any of the CI animals after day 49, reasons for the reduced egg output in the CI goats during the middle part of the trickle challenge period may be due to the presence of higher proportions of non-patent early L_4 larvae (EL_4) in the intestinal mucosa of the CI goats when compared to the CI lambs.

EL_4 accounted for 24.4% and 15.8% of the total population in CC yearlings and CC kids compared to 8.3% in CC lambs, whilst a similar picture was also evident in the CI group, with EL_4 accounting for 58.8, 43.3 and 28.8% of the total populations recovered from the CI yearlings, kids and lambs. Since a high degree of retardation also occurred in the CC kids, this is unlikely to be attributable to an acquired immunity, but may be linked to the biochemical or physiological conditions prevailing in the abomasa. In this respect, the pHs in goat abomasa were found to be 70% higher in the CI animals and at least 40% higher in the CC goats compared to similarly challenged lambs. The reasons underlying this are currently unknown, since there is little previous data available for abomasal pH values in goats undergoing either natural or experimental nematode challenges. The goats in the current study were all housed and raised exclusively on lamb-formulated rations, which may have

contributed to the production of these high abomasal pHs. Further experiments involving similarly managed, worm-free control animals would be required to confirm this. Another possibility is that the strain of parasite used in this experiment was isolated and maintained in sheep which may have accounted for some of the differences observed in nematode fecundity and development between the species. However, this is unlikely, since the same strain was used in the previous study (Huntley *et al.*, 1995), where challenged does were found to have higher egg counts and higher worm burdens than infected ewes.

Previous studies investigating the development of immune responses to *T. circumcincta* demonstrated that one of the first expressions of acquired immunity in young lambs was a reduction in the rate of development of incoming larvae (Seaton *et al.*, 1989a, b). Other studies have also demonstrated reductions in the fecundity of adult females during the development of immunity to *T. colubriformis* and *H. contortus* infections in lambs (Dineen and Windon, 1980; Gill *et al.*, 1991). Although both these changes were apparent in the CI yearlings and kids, at post mortem, the CI lambs contained significantly fewer worms than the CI kids, marginally less than the CI yearlings as well as lower L_3 establishment ratios than either age group of CI goats. Additionally, there was also a trend, although not significant, for higher initial establishment of L_3 in the CC kids following the primary challenge seven days previously.

In general, these findings are consistent with the previous observations of Huntley *et al.* (1995), where it was demonstrated that adult does, although they had extensive prior exposure to natural nematode infections, had considerably larger abomasal worm burdens than worm naive lambs following experimental challenge. As in the current experiment, there was also evidence of immunologically and/or physiologically mediated effects upon larval development since the does retained high proportions of their *T. circumcincta* burden as L_4. Unfortunately, abomasal pH values

were not recorded. An additional finding in this current experiment was that previously worm-free kids undergoing both primary (CC) and secondary (CI) challenge regimes also exhibited a greater degree of larval retardation than the similarly aged lambs. Overall, the findings from both experiments suggest important differences in the mechanisms that regulate the kinetics of abomasal infections in the two species. On the one hand, responses in kids, yearlings and does appear to retard larval growth without reducing total worm numbers whereas on the other, responses in lambs and ewes allow the development of established worms to the L_5/adult stage but at the same time, restrict establishment and/or increase expulsion of adult worms.

The wide variations in the abomasal worm burdens recovered from the CI yearlings when compared with the CI kids also suggests the presence of an age-related acquired response that becomes more effective at rejecting nematodes in certain individual goats as they become older. The genetic basis of this variability in the individual responses of older goats has been confirmed previously (Patterson, Jackson, Huntley, Stevenson, Jones and Russel, 1995). However, even at the age of eighteen months, their ability to regulate the size of the total worm population was not as well developed as that of six month old lambs.

Elevated numbers of intestinal MMCs and intra-epithelial GLs are a characteristic feature of helminthiasis in ruminants (Miller, 1984). As might be expected there were significant differences between the numbers of MMC and total mast cells (combined MMC and GL counts, see Chapters 3 and 4) between the CI and CC groups. Total mast cell numbers in the CC and CI infected yearling and lamb tissues were found to be inversely related to the total abomasal worm numbers but in the kids there was either no correlation (CC kids) or a positive relationship (CI kids). This suggests that, while the presence of MMCs and GLs may reflect the ability to regulate worm numbers during primary and secondary infections in the lambs and older goats, this was not the case in infected kids.

Significantly greater proportions of the total mast cells in the CI yearlings and kids were also found to be GLs (61 and 47% respectively), compared to the CI infected lambs (9.4%). These findings are again in agreement with those of Huntley *et al.*, (1995) who demonstrated that significantly greater proportions of GL were present in adult does compared to adult ewes, following a natural and artificial challenge regime. The presence of elevated numbers of GLs in the gut mucosa has been closely correlated with increased resistance to nematode infections in sheep (O' Sullivan and Donald, 1973, Gregg *et al.*, 1978; Gill *et al.*, 1991, see general introduction), but given the high numbers of worms present in the abomasa of adult, yearling and kid goats, this is clearly not the case in this species. GLs have been shown to be degranulated MMCs in sheep (Huntley *et al.*, 1984) whilst in the goat, the presence of low levels of GMCP within the cytoplasmic globules of some GL (Chapter 3), suggests their mast cell origin in this species also. As such, their presence may be indicative of increased mast cell activation, which has been associated with type I hypersensitivity reactions that accompany the expulsion of nematodes from the intestines of immune animals (Miller, 1996a). Therefore, the finding that the secondary infected yearlings and kids were unable to reduce their total worm burdens to the equivalent levels found in the secondary infected lambs, despite the presence of significantly higher numbers of GL, is a further indication of deficiencies in mast cell-mediated expulsive mechanisms in the goat.

Cell counts from tissue sections stained with anti-GMCP demonstrated increased mean numbers of positive cells in the CI group compared to the CC group although the increase was not as significant as that found in equivalent histochemical stained tissue sections. Regression analysis demonstrated a higher correlation between total mast cell numbers and the cells staining positive with anti-GMCP in the tissues of CC animals compared with the tissues of CI animals. This lack of correlation in the trickle-challenged animals was probably due to the presence of much larger

numbers of poorly staining GL and degranulated MMC in the tissues of the CI yearlings and kids (see Chapter 3 and Chapter 4) possibly as a result of chronic MMC stimulation by worm antigens throughout the challenge period. This was borne out by the finding that equivalent correlations on counts for the CC and CI lamb tissues alone, where tissue protease concentrations were much higher (see below) and the GL numbers significantly lower, remained almost constant at $R^2 = 0.85$ and 0.80 respectively ($p < 0.001$ in both cases).

ELISA results for tissue mast cell protease content using antibodies that are cross reactive for both GMCP and SMCP (Chapter 3), demonstrated a significant increase in the SMCP content of the CI lambs compared to the CC lambs. This indicates that upregulation of SMCP production occurred in the lambs during the trickle challenge period. This upregulation did not appear to be matched in the equivalently infected yearling and kid tissues, which contained significantly less GMCP in both the CC and CI animals. This could indicate a fundamental deficiency in protease synthesis by the goat mast cells, although species differences in the kinetics and quantities of protease synthesis and/or release from the goat and lamb mast cells following challenge cannot be ruled out. For instance, earlier or more sustained release of GMCP from goat mast cells may have resulted in comparatively greater depletion of the enzyme from the goat tissues by the time they were collected and the enzyme concentrations assayed. Moreover, if incoming larvae are important for triggering mast cell degranulation and GL formation, then these animals were all sacrificed seven days after their last exposure to this source of antigens. Therefore, it is also possible that mast cell protease production is maintained for longer in the lambs than the goats through continued synthesis and storage in MMCs rather than in GL. If so, this may also have contributed to the elevated residual SMCP concentrations due to the presence of relatively higher numbers of MMC in the lamb tissues. Further experiments aimed at investigating these two possibilities are

warranted, these might include time course studies designed to examine comparative changes in tissue protease concentrations during the 7 to 10 day period after a primary or secondary larval challenge.

A further interesting finding was the significant increase in mast cell protease concentrations in the CI lamb but not the CI goat jejunal tissues. This occurred despite the predeliction of *T. circumcincta* for the abomasum, indicating that the factors regulating SMCP synthesis in lambs may also influence MMCs at sites not directly in contact with nematodes. Furthermore, mean cell counts for MMC and GL in the jejunum (results not shown), did not demonstrate any significant increases when comparing CC and CI infected animals from either species. The significance of this remains to be fully determined, but suggests that increased synthesis and/or storage of protease is induced in individual lamb but not goat jejunal MMCs following a period of continuous challenge or secondary challenge with abomasal parasites.

Increased numbers of peripheral and tissue eosinophils are a characteristic response to intestinal parasitism (Jones, 1993), and have been implicated in host resistance to nematode infections (Buddle *et al.*, 1992), although their exact role in this context remains controversial (reviewed Rothwell, 1989). The peripheral blood eosinophil results obtained in this experiment were found to be highly variable, with no significant differences observed between the CC and CI groups. Likewise, there were no significant differences in the responses shown between the goats, yearlings and lambs within either group, although for reasons yet to be determined, mean counts were found to be higher in the CC and CI kids. Previous results from ewes and does undergoing natural challenge at pasture suggests that continuous antigenic challenge is required to maintain circulating eosinophil numbers, since blood eosinophil levels decline significantly after housing and anthelmintic treatment (Huntley *et al.*, 1995). However, in the present study, artificial challenge over a period of 49 days did not appear to increase the numbers of peripheral circulating

eosinophils significantly in the CI animals. Whether higher experimental challenge levels and/or dosing for longer periods are therefore required to induce blood eosinophilia remains to be determined.

In the case of the tissue eosinophil numbers present in the abomasal tissues at post mortem, significant differences between the CC and CI infected groups were noted in the kids only. There were no consistent relationships detected between the numbers of tissue eosinophils and the total numbers of abomasal worms or the proportions of worms recovered as L_4 for animals in either group. These results would therefore suggest that tissue eosinophils are unlikely to be directly responsible for the differences in the established L_4 and total worm burdens found between the primary or secondary infected yearlings, kids and lambs.

Results from the serum IgE time course studies demonstrated possible age and challenge dose-dependent influences on the production of total serum IgE in the two species. Despite a high degree of variability amongst individuals, the CI yearlings generated consistently higher mean IgE responses to the trickle challenge than either the CI lambs or kids. This suggests that the younger animals are less able to produce IgE responses in the face of moderate levels of larval challenge. This apparent inability was overcome by administering the larger challenge at day 49, which generated a sharp increase in the IgE response in the CC animals between five and seven days post challenge. The larger dose also produced elevations in the mean total serum IgE responses of the CI animals. suggesting a challenge-dose dependency for both primary and secondary IgE responses. Interestingly, the secondary responses shown by the CI animals did not start any earlier and were not significantly higher than the responses shown by the CC animals. Indeed, in the kids, the CC response was significantly higher than the CI response. This suggests that an anamnestic rise in total IgE levels following secondary nematode infections does not occur in goats and lambs, which agrees with previous findings in adult sheep (J. Huntley, personal communication).

However, it should be emphasised that in this study, it was not possible to measure specific IgE antibody levels, and it is recognised that total IgE concentrations may not reflect the true IgE antibody status of these animals (Jarrett *et al.*, 1980). Further work into the development of assays able to detect the presence of parasite-specific IgE is therefore required, to confirm whether the findings presented here can be fully attributed to the host's response to gastrointestinal nematodes.

Overall, the findings in this experiment confirm and extend the observations made by Huntley *et al.* (1995) regarding the inability of goats to regulate their gastro-intestinal worm burdens when compared to equivalently challenged sheep. Moreover, they also provide further evidence for significant differences in mast cell functional activity in both species, particularly with regard to the extremely low levels of GMCP and high numbers of GL present in the goat gastro-intestinal tissues. The next two chapters will therefore focus on the functional responses shown by goat mast cells in isolation. This will be achieved by generating and characterising goat BMMC and by purifying goat MMC and GL from parasitised intestinal tissues before analysing their respective activities following *in vitro* exposure to nematode antigen preparations.

CHAPTER 7

THE EFFECTS OF RECOMBINANT OVINE INTERLEUKIN-3 AND RECOMBINANT OVINE STEM CELL FACTOR ON THE GROWTH AND MEDIATOR EXPRESSION OF CAPRINE AND OVINE BONE MARROW DERIVED MAST CELLS.

7.1 Introduction

The *in vitro* production of cytokine-dependent mast cells from haemopoietic and lymphoid tissue has been demonstrated in a number of species, including mice (Razin *et al.*, 1984), rats (Haig *et al.* 1988a) and humans (Furitsu, Saito, Dvorak, Schwartz, Irani, Burdick, Ishizaka and Ishizaka, 1989). Sources of mast cell stimulatory factors were initially derived from fibroblast monolayers and conditioned medium harvested from the supernatants of murine WEHI-3 tumour cells or mitogen-activated T cells (Razin *et al.* 1984, Levi-Schaeffer, Dayton, Austen, Hein, Caulfield, Gravallese, Liu and Stevens, 1987). Conditioned medium derived from conacavalin A stimulated mesenteric lymph node cells has also been used to grow mast cells successfully from rat and sheep bone marrow (Haig *et al.* 1983; Haig *et al.*, 1988b). More recently, advances in cytokine gene cloning have provided access to recombinant cytokines, allowing their individual and combined effects to be studied *in vitro*. Both IL-3 and SCF stimulate the development and proliferation of mast cells in mice (Razin *et al.* 1984, Zsebo *et al.* 1990a, Rennick *et al.*, 1995), rats (Haig *et al.*, 1988a, Haig *et al.* 1994) and the basophil or mast cell lineages in humans (Saito *et al.*, 1988; Durand *et al.*, 1994, Metcalfe *et al.*, 1995). In sheep, sequencing of the gene for ovine interleukin 3 (McInnes *et al.* 1993) and the cDNA for ovine stem cell factor (McInnes, unpublished, EMBL No.Z50743) has enabled expression of their respective recombinant protein products for the work presented here.

The objectives of the study described in this Chapter were to :

a) Determine the effects of rOvIL-3 and rOvSCF either alone or in combination on the generation of mast cells from caprine and ovine haemopoietic precursor cells.

b) Examine the effects of these cytokines on the growth kinetics, longevity and granule-associated mediator content of these cell cultures.

c) Look for significant inter-species differences in mast cell granule content.

d) Produce cultures containing sufficient numbers of viable mast cells for future nematode allergen-induced mediator release studies.

7.2 Materials and methods

7.2.1 Animals

Suffolk cross lambs aged six - twelve months and Scottish cashmere goats aged six months - four years were used in all experiments. The sheep were bred at the MRI, and the goats bought from Sourhope farm, Kelso, Roxburghshire, Scotland. All animals used had been previously exposed to nematode parasites whilst maintained on pasture.

7.2.2 Culture techniques

Preparation of rOvIL-3 and rOvSCF

Cell supernatants containing these cytokines were generously provided by Dr D. Haig and Dr. G. Entrican having been prepared, as described by McInnes *et al.* (1993), for the production of rOvIL-3. Stable expression of rOvIl-3 and rOvSCF was achieved in Chinese Hamster Ovary (CHO) cells using the Celltech® pEE14 vector (Cockett, Bebbington and Yarrington, 1990). The gene for rOvIL-3 (McInnes *et al.* 1993) and the cDNA for the soluble form of rOvSCF (McInnes, unpublished, EMBL. No.Z50743) were ligated into separate vectors adjacent to the human cytomegalovirus immediate early promotor sequence. The vector contains the glutamine synthetase (GS) gene as an amplifiable marker in CHO cells. After transfection of ligated pEE14 IL-3/SCF plasmids into CHO cells, transfectants expressing elevated levels of GS were selected in the presence of 25 - 250 μM methionine sulphoximine (MSX; Sigma Cat. No. M 5379). Expression of rOvIL-3 and rOvSCF mRNA was checked by Northern blotting, and recombinant protein

expression by gel electrophoresis. Batches of supernatant were collected and assayed for biological activity in 24 well plate bone marrow cultures as described below.

Bone marrow cultures

Sternal bone marrow cells were isolated and established in culture as described in 2.10.1. Cells were adjusted to 2 or 3 x 10^5 cells per ml IMDM + 2-ME +10%FCS + P/S and aliquoted into sterile 24 well plates for dose response testing of the cytokine supernatants, or 75-225 cm^2 flasks for time course studies.

Dose response studies

For dose response studies to determine optimal cytokine concentrations, 1ml aliquots per well of isolated bone marrow cells at 2 x 10^5 cells per ml were set up in triplicate sets of wells. To each set was added dilutions of rOvIL-3 alone or a combination of rOvIL-3 and dilutions of rOvSCF. The rOvIL-3 and rOvSCF supernatants were diluted in IMDM + 10%FCS + P/S + 2-ME to give a final range of 10^{-1} to 10^{-6} v/v rOvIL-3 supernatant for the rOvIL-3 titration and 10^{-1} to 10^{-4} v/v rOvSCF alone or in the presence of an optimised dilution of 10^{-2} rOvIL-3 for the rOvSCF titration. For each experiment, the same pattern was repeated in 2 further plates to allow harvesting at 3 separate time points. A seventh set of triplicate wells was also set up on each plate, containing IMDM and 10^{-1} v/v non-transfected CHO supernatant (IMDM + CHO) as a negative control. All plates were incubated and fed with fresh cytokines and IMDM as described in Chapter 2.10.2.

Individual plates were harvested for their supernatants and cells at 3 separate time points (days 6, 12 and 18 for rOvIL-3 titrations and days 7, 14 and 21 for rOvSCF titrations) as described in Chapter 2.10.3.

Time course studies

For time course studies, isolated bone marrow cells were set up at 3 x 10^5 cells per ml in IMDM + 10%FCS + P/S + 2-ME, containing optimal concentrations of rOvIL-3 and rOvSCF supernatant in 75-225 cm^3 sterile tissue culture flasks (Corning,

USA). The initial culture volume was noted, and the flasks incubated under the same conditions as the 24 well plates. Every 48-72 hours supernatant and cells were harvested and the cultures fed with fresh cytokines as described in 2.10.2 and 2.10.3. At the same time, the total culture volume was adjusted to maintain the cells at < 3-5 x 10^5 cells per ml.

7.2.3 Enumeration and staining of BMMC

Cytosmear preparations were prepared as described in Chapter 2.8.2. The slides were either stained immediately with Leishman's (Gurr) for immediate assessment of the cultured cell populations (see Chapter 8; Fig. 8.1c), or fixed in 4% paraformaldehyde PBS as described in Chapter 2.8.6 and Chapter 2.8.2. Fixed slides were subsequently stained for the presence of mast cells using rabbit polyclonal antibodies raised against goat mast cell protease (Chapter 3) as described in Chapter 2.8.7. Positive cells on the cytosmears (see Chapter 8; Fig. 8.1d) were counted as described in Chapter 2.8.9

7.2.4 Mast cell enzyme assays

Culture supernatants and cell extracts were prepared as described in Chapter 2.10.4. The supernatants and extracts were tested for their GMCP, SMCP, aryl-sulphatase and β-hexosaminidase content as described in Chapter 2.12.

7.2.3 Statistical analysis

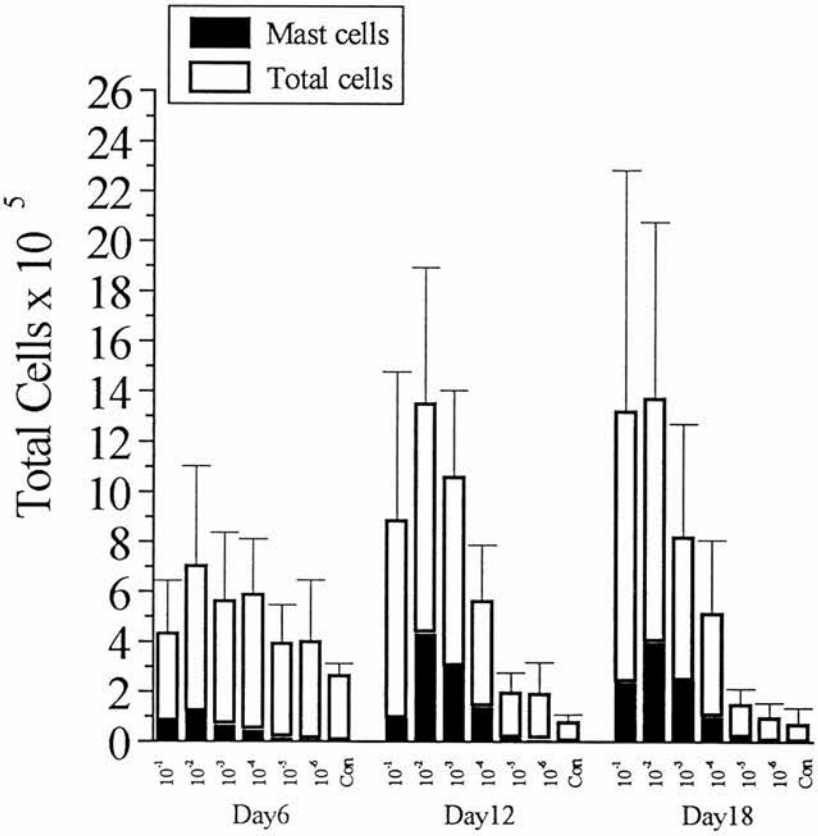
Group mean results obtained from 3 separate cultures or more were compared using a paired Students T-test as described in 2.14.1. Graphed data (except for Fig. 7.3) is plotted as the mean result (±SD) of at least 3 observations from a minimum of two separate cultures.

7.3 Results

7.3.1 Cytokine dose response studies

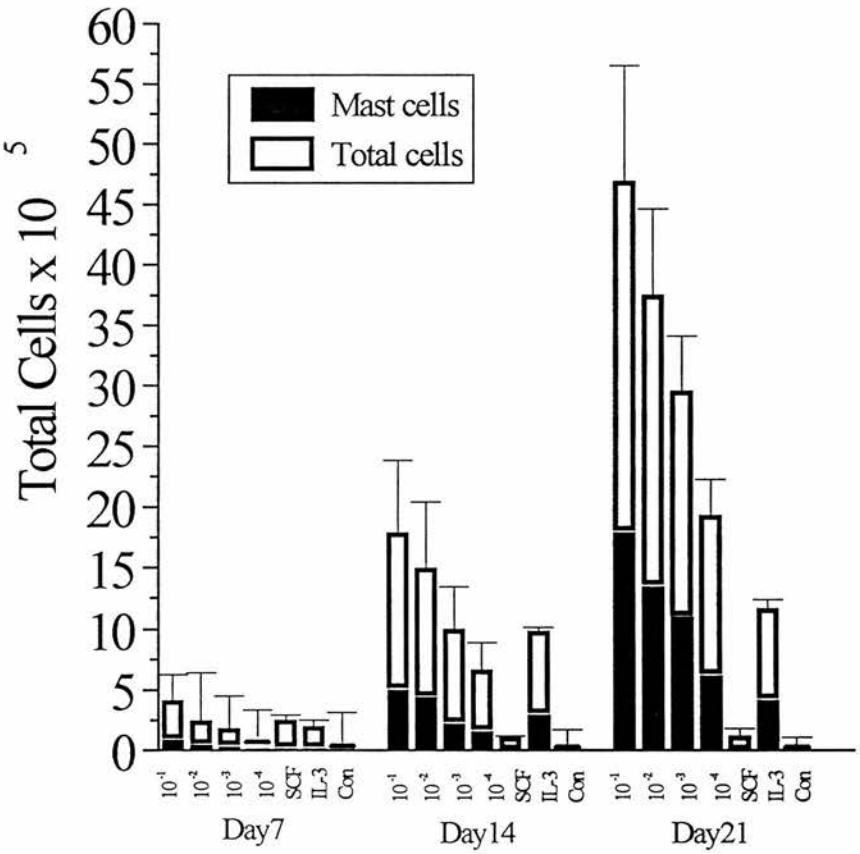
Figures 7.1 and 7.2 show the total cell numbers for goat cells grown in 10^{-1} to 10^{-6} rOvIL-3 alone (Fig. 7.1) or 10^{-1} to 10^{-4} rOvSCF with or without rOvIL-3 at 10^{-2} (Fig. 7.2). The results demonstrated a dose response effect on total cell proliferation when rOvIL-3 alone was used at 10^{-2} to 10^{-6} and rOvSCF at 10^{-1} to 10^{-4} in combination with 10^{-2} rOvIL-3. Significantly larger numbers of cells were produced by day 21 (Fig. 7.2) in cultures incorporating both cytokines at 10^{-1} v/v when compared with cultures containing 10^{-2} rOvIL-3 alone, 10^{-2} rOvSCF alone or IMDM + CHO.

Figures 7.1 and 7.2 also show total mast cell numbers calculated from the percentage of cells reacting with antibody to GMCP. In the rOvIL-3 dose response cultures (Fig. 7.1), cells grown in 10^{-2} rOvIL-3 produced up to 50% GMCP-positive mast cells by day12 . This response appeared to be dose dependent at all three time points for cells grown in rOvIL-3 at 10^{-2} to10^{-6} although when 10^{-1} rOvIL-3 was used, significantly fewer cells ($p<0.01$) stained positive at both day12 and day 18. In the rOvSCF dose response cultures (Fig. 7.2), cells grown in 10^{-1} rOvSCF with 10^{-2} rOvIL-3 produced up to 65% mast cells by day 21. This response was dose dependent for 10^{-1} to 10^{-4} rOvSCF at all three time points. In contrast, cells grown in rOvSCF alone (Fig. 7.2) showed no significant increase in mast cell numbers when compared with the IMDM + CHO control cultures. ELISA results for supernatants harvested from all cultures were below the levels of assay detection (< 0.5 ng/ml).



(±SD)

Fig.7.1Mean total and GMCP-positive mast cell numbers for goat bone marrow cells set up in 10^{-1} to 10^{-6} dilutions of rOvIL-3 or IMDM + 10^{-1} CHO supernatant. 10^{-1}-10^{-6} = rOvIL-3 dilutions; Con = IMDM + 10^{-1} non-transfected CHO Supernatant



(±SD)

Fig7.2 Mean total and GMCP-positive mast cell numbers for goat bone marrow cells set up in 10^{-1} to 10^{-4} dilutions of rOvSCF with 10^{-2} rOvIL-3, 10^{-2} SCF only, 10^{-2} rOvIL-3 only or IMDM + 10-1 CHO supernatant . 10^{-1} - 10^{-4} = rOvSCF + 10^{-2} rOvIL-3; SCF = 10^{-2} rOvSCF alone; IL-3 = 10^{-2} rOvIL-3 alone; Con = IMDM + 10^{-1} non-transfected CHO supernatant

Since no significant differences ($p>0.05$) were detected in both the total and mast cell counts for cells grown in 10^{-2} rOvSCF compared with 10^{-1} rOvSCF, whilst the use of 10^{-1} rOvIL-3 suppressed mast cell numbers, both cytokines were used at 10^{-2} v/v in the subsequent time response experiments.

7.3.2 Cytokine time course studies

Figure 7.3 shows the total cell numbers obtained from two typical flask cultures maintained over 47 days. The cells were fed with 10^{-2} rOvIL-3 alone or 10^{-2} rOvIL-3 and 10^{-2} rOvSCF. Cells maintained in rOvIL-3 alone showed only a moderate rise in total cell numbers with a peak of 7×10^7 total cells on day 7. In contrast, the addition of 10^{-2} rOvSCF resulted in a marked rise in cell numbers beginning on day 3 with a peak of 2.6×10^8 cells by day 17. Total cell numbers dropped earlier in the cultures stimulated with rOvIL-3 alone, so that on day 27 very few cells were detected. The remaining cells had the appearance of monocyte/ macrophages on Leishman stained cytosmears and were GMCP-negative. The rOvIL-3 and rOvSCF fed cells also declined in numbers after day 17, but after 3 weeks growth, the cell numbers stabilised, and the cultures could then be maintained for up to 7 weeks before degenerating.

Figure 7.4 shows the mean (n=3) percentage totals for GMCP-positive staining cells obtained from time response cultures. In the early stages (day2 - day 5), more cells grown in both 10^{-2} rOvIL-3 and 10^{-2} rOvSCF stained positive when compared with those grown in 10^{-2} IL-3 alone (results only significant ($p<0.05$) at d2). Thereafter, the percentage positive cells in the rOvIL-3 alone cultures increased rapidly, overtaking the rOvIL-3 + rOvSCF cultures by day 7. Subsequently, the percentage of GMCP-positive cells grown in rOvIL-3 alone declined over the next 12 days, and by day 31 there were no positive cells left in the culture. In contrast, the

percentage of GMCP-positive cells in the rOvIL-3 + rOvSCF cultures increased to produce peak numbers of mast cells by day 12, but following a slight decline between day 12 and day 14, the numbers of positive cells stabilised so that by day 42, there were still GMCP-positive cells present. Cells grown in 10^{-2} rOvSCF alone showed no significant increase in the numbers of GMCP-positive cells in the cultures from day 2 onwards. At day 7 and day 12 there were more cells positive for GMCP in these cultures when compared to equivalent control cultures grown in IMDM + 10^{-1}CHO alone. However, by day 18 there was no protease detected in any of the remaining cells which were again identified predominantly as macrophages from their morphological appearance on Leishman stained cytosmears

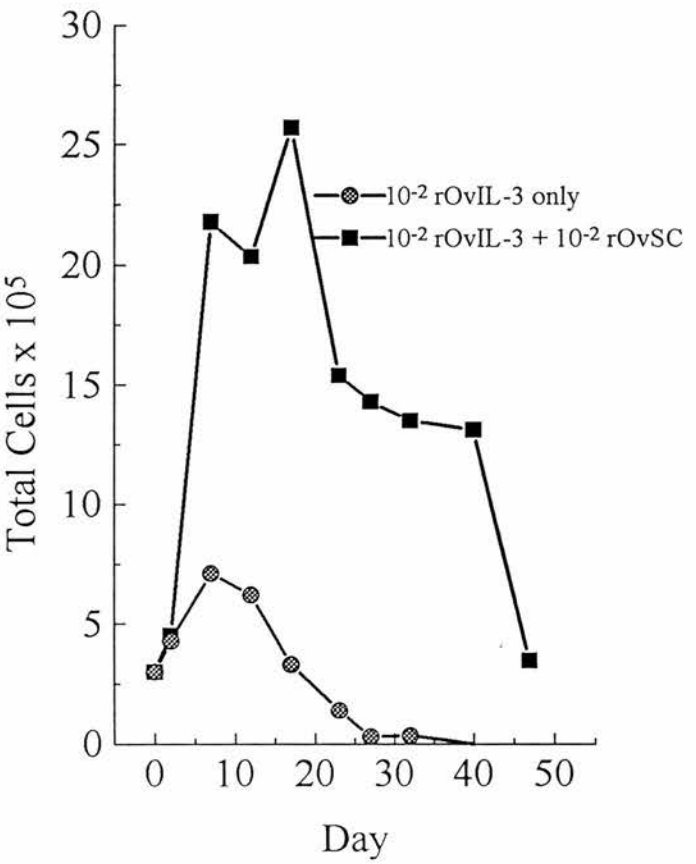


Fig.7.3 Total cell numbers for flask grown goat bone marrow cells fed with 10^{-2} rOvIL-3 and 10^{-2} rOvSCF or 10^{-2} rOvIL-3 alone.

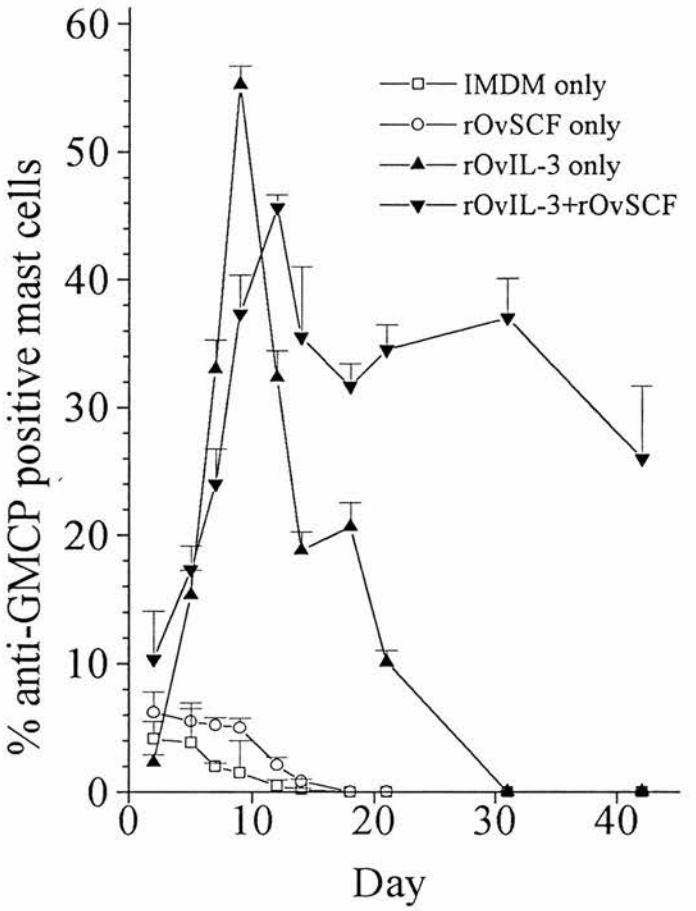


Fig. 7.4 Mean (±SD) percentages (n=3) of the goat bone marrow time response culture cells that react positively with antibody to GMCP

7.3.3 Enzyme concentrations

1) Mast cell proteases

Figure 7.5 shows the mean SMCP/GMCP content in ng per 10^6 mast cells for lysed cell pellets taken from 4 time response cultures per species. Day 0 sheep bone marrow samples contained significantly more protease than the goat day 0 samples. Following an initial (day 0 to day 5) decline in protease levels present in the sheep cells, the protease concentrations in the cell pellets in all cultures increased slowly with no significant differences noted between the species or combinations of cytokines used.

After day 7, SMCP levels in the sheep cultures increased significantly compared to the goat cultures giving a peak value of 13.82 ±1.6 ng per 10^6 mast cells for rOvIL-3 cultures and 17.52 ±7.78 ng per 10^6 mast cells for the rOvIL-3 and rOvSCF cultures. In the goat cultures, equivalent concentrations of GMCP were 5.42 ±1.7ng per 10^6 mast cells and 5.0 ±3.05 ng per 10^6 mast cells for rOvIL-3, or rOvIL-3 and rOvSCF fed cultures respectively. No significant differences were noted in the protease content of cultures from either species when comparing cells grown in rOvIL-3 alone with cells grown in rOvIL-3 and rOvSCF during the first 3 weeks. However, from day 21 onwards, there was a rapid decline in the protease content of cultures from both species fed with rOvIL-3 alone, with no protease detected after 4 weeks. In contrast, by day 35 the sheep and goat cells grown in rOvIL-3 and rOvSCF still contained 7.46 ±3.6 and 4.73 ±0.77 ng per 10^6 mast cells respectively.

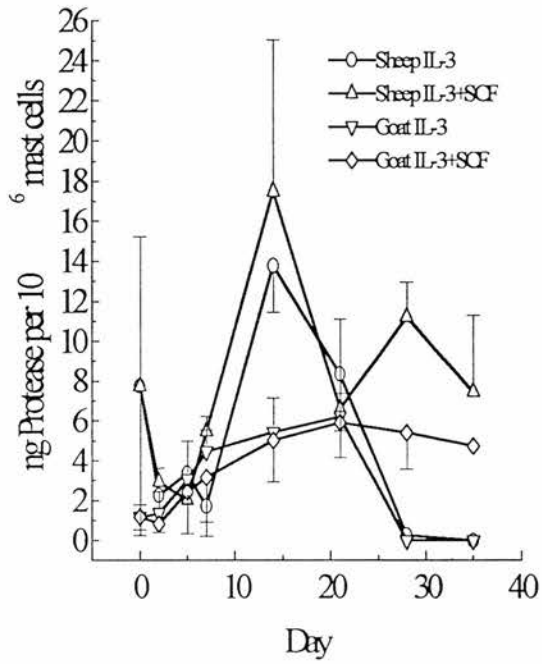


Fig.7.5 SMCP/GMCP content of sheep and goat bone marrow cells grown in 10^{-2} rOvIL-3 and 10^{-2} rOvSCF or 10^{-2} rOvIL-3 alone (expressed as ng protease/10^6 mast cells).

2) β-Hexosaminidase

Figure 7.6 shows the results for β-hexosaminidase content defined as units of activity per 10^6 mast cells. Enzyme activity was minimal until day two for all cultures, which corresponded with the appearance of significant numbers of immature mast cells on Leishman stained cytosmears. Thereafter, the sheep and goat cells grown in IL-3 alone show peak activity at day 14 (8.58 ±3.5 and 4.32 ±0.4 units per 10^6 mast cells respectively) before declining to zero by day 35. The cells grown in the presence of rOvSCF continued to increase their β-hexosaminidase content until day 28, with the goat cells containing significantly more than the sheep cells at this time point. However, by day 35, the β-hexosaminidase content of the goat cells had decreased to 6±5.0 units per 10^6 mast cells which was not significantly different to the sheep cells which contained 4.5 ±0.3 units per 10^6 mast cells.

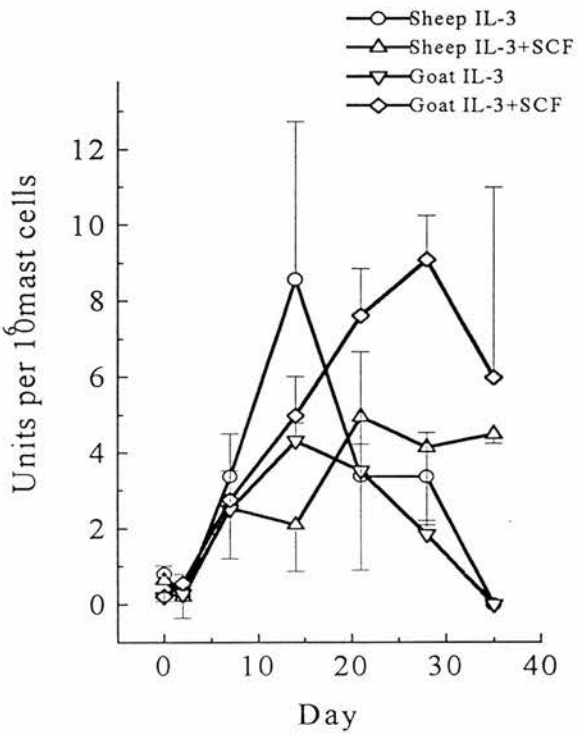


Fig.7.6 β-hexosaminidase content of sheep and goat bone marrow cells grown in 10^{-2} rOvIL-3 and 10^{-2} rOvSCF or 10^{-2} rOvIL-3 alone (expressed as units/10^6 mast cells)

3) Aryl-sulphatase

Figure 7.7 shows the results for aryl-sulphatase content defined as μg aryl-sulphatase per 10^6 mast cells. Throughout the culture period, the sheep cells contained significantly more ($P < 0.05$) aryl-sulphatase than the goat cells. There were no significant differences observed in the mediator content of cells cultured in rOvIL-3 alone when compared with those cultured in rOvIL-3 and rOvSCF during the first two weeks of culture. However, after 3 weeks, the mediator content of the cells from both species grown in IL-3 alone declined, so that by the 4th week they contained approximately 15% of the aryl-sulphatase content of the equivalent cells grown in rOvIL-3 and rOvSCF.

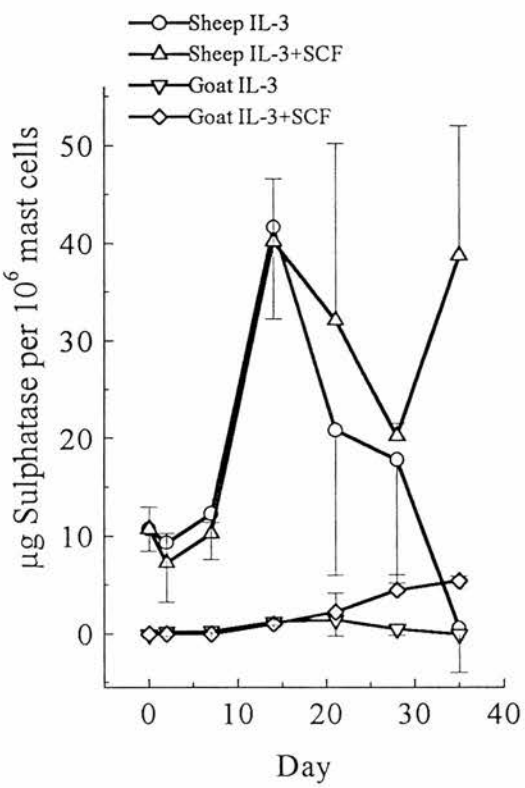


Fig.7.7 Aryl-sulphatase content of sheep and goat bone marrow cells grown in 10^{-2} rOvIL-3 and 10^{-2} rOvSCF or 10^{-2} rOvIL-3 alone (expressed as μg/10^6 mast cells)

7.4 Discussion

Previous work by McInnes, Haig and Logan (1993) and Sture (1996) demonstrated that rOvIL-3 alone was capable of producing mast cells from ovine sternal haemopoietic cells. However the resulting cultures were of limited lifespan (three to four weeks), and the cells generated appeared immature in terms of morphology and mediator content when compared with mast cells isolated *ex vivo* from sheep intestinal tissues (Huntley, 1991; Huntley *et al.* 1992). Pilot studies, carried out prior to the work presented here, also demonstrated that rOvIL-3 alone was able to stimulate the development and growth of caprine BMMC.

A major result of this study was that optimal amounts of rOvSCF, in combination with rOvIL-3, significantly increased the total cell numbers produced in both ovine and caprine bone marrow cultures. Both cytokines also increased the longevity of the cultures for at least 3-4 weeks beyond the normal lifespan of cultures generated in rOvIL-3 alone. These responses were only seen in cells grown in a combination of rOvSCF and rOvIL-3, and not in cells cultured with either cytokine alone indicating that the effect was dependent on synergy between the two cytokines. The results differ from previous data obtained from rat cultures (Haig *et al.*, 1994), which demonstrated that recombinant SCF alone promoted the development cultured mast cells in this species. However, the presence of a synergistic action of rOvSCF and rOvIL-3 in rat and human mast cells has been demonstrated (Haig *et al.*, 1994, Metcalfe *et al.*, 1995), whilst a role has also been suggested for SCF in promoting the survival of cultured human mast cells by suppressing apoptosis (Iemura, Tsai, Ando, Wershil and Galli, 1994; Galli *et al.* 1995). The prolonged survival of ovine and caprine BMMC achieved by the addition of rOvSCF in the present study may, in part, be due to the ability of rOvSCF to suppress an apoptotic mechanism which is still functioning in cells that have been cultured in rOvIL-3 alone.

There were no significant differences in the mediator content of cells from either species grown in rOvIL-3 alone when compared with cells grown in rOvIL-3 and rOvSCF, which was also at variance with previous data obtained for rat (Haig *et al.* 1994). In these studies, the RMCP-II content of BMMC maintained in SCF was significantly less than that of cells maintained in either IL-3 or LNCM alone whilst there was also an increase in expression of the CTMC-associated protease RMCP-I. RMCP-II could also be detected in the supernatants of BMMC, especially when BMMC numbers were increasing rapidly in SCF with or without the presence of IL-3 or LNCM. This indicated that SCF not only regulates the growth of rat BMMC, but it also influences the production and release of protease mediators. SCF has been shown to induce directly SCF receptor-dependent mast cell mediator release in mice and humans (Galli *et al.*. 1995), but equivalent findings were not demonstrable in this study. Supernatant samples taken from the dose response and time course cultures were all found to be negative when tested by ELISA (< 0.5 ng protease/ml). However, these results should perhaps be treated with caution since the culture techniques used required relatively frequent changing of medium in the dose response experiments, and the addition of large volumes of medium to maintain cell populations at 3-5 x 10^5 cells per ml in the time course experiments. This, combined with the low levels of mediator present in these cultured cells, may have resulted in the dilution of any released protease to below detectable levels.

On examining the time course total cell count data, it can be seen that over the period of maximal expansion in cell numbers (between day 2 and day 7), the mediator content of the harvested pellets remained low, indicating possible immaturity of the expanding population of cells.. This profile changed as cell proliferation slowed by day 12 and the mediator concentrations generally increased indicating that a maturation process was occurring. Thereafter, cells that were maintained in rOvIL-3 alone declined in numbers from 2-3 weeks onwards, with a concomitant decrease in

their mediator content as they degenerated. In contrast, cells grown in both rOvIL-3 and rOvSCF continued to survive for a further 2-3 weeks, with relatively stable concentrations of aryl sulphatase, protease and β-hexosaminidase.

Significant differences were noted in the mediator content of sheep bone marrow derived cells when compared to their goat equivalents. The goat cells generally contained less GMCP, β-hexosaminidase (with the exception of goat BMMC grown in rOvIL-3 and rOvSCF) and arylsulfatase after the first five days of culture. Whether these differences are due to incomplete biological cross reactivity of the ovine cytokines on caprine cells, or fundamental differences in mediator synthesis in caprine and ovine BMMC, has not been established. However, evidence in support of the latter has come from observations that goat tissues and gastro-intestinal derived isolated MMC contain less mast cell protease than their sheep equivalents (Huntley *et al.* 1995, Chapter 3, Chapter 6 and Chapter 8).

The significantly lower concentrations of aryl-sulphatase present in the goat BMMC cultures were also remarkable. Like β-hexosaminidase, this acid hydrolase enzyme has been found in mast cells secretory granules from a variety of species including rats, humans and sheep (Schwartz and Austen, 1981; Huntley, 1991; Huntley, *et al.* 1992). However, they have been associated with the degradation of ingested glycosaminoglycans, glycoproteins and glycolipids in intracellular phagolysosomes. Phagocytosis has been demonstrated in rat mast cells (Vranian, Conrad and Ruddy, 1981), but their function following release into the extracellular environment from secretory granules is still not understood. Both enzymes have an acidic pH optimum indicating that secretion into gastric or inflammatory acidic environments would be required to permit expression of maximal activity (Schwartz and Austen, 1984). Such circumstances may occur following release into the abomasum during gastro-intestinal responses to nematode parasites. As yet, no studies have been performed to examine the role of these enzymes *in vivo* in

parasitised ruminants. However, the significant lack of aryl-sulphatase in goat BMMC indicates a further mediator deficiency associated with these cells which may have relevance to MMC responses associated with the regulation of gastro-intestinal worm burdens.

In terms of morphology and mediator content, BMMC generated with rOvIL-3 and rOvSCF from both species appear immature when compared with isolated MMC (see Chapter 8 Figures 8.1a and 8.1c) or cells grown using ovine LNCM (Huntley, 1991). This would indicate that there are other factors required for full mast cell maturation and granule-associated mediator expression. These may include the cytokines IL-4, IL-9 and IL-10 which have been shown to affect murine and human mast cell development (Metcalfe *et al.*, 1995, Renauld *et al.*, 1995, Rennick *et al.*, 1995) but as yet, ruminant equivalents of these reagents are not available for use in *in vitro* studies.

The following chapter extends these findings by comparing the morphological, biochemical and ultrastructural characteristics of goat and sheep BMMC with MMC and GL isolated from goat intestinal tissues. This is followed by the results of studies examining their functional responses to stimulation with synthetic secretogogues and nematode antigen preparations.

CHAPTER 8

THE BIOCHEMICAL AND FUNCTIONAL CHARACTERISATION OF ISOLATED GOAT MMC AND *IN VITRO* DERIVED BMMC: ANALYSING THEIR RESPONSES TO STIMULATION WITH SECRETOGOGUES AND CRUDE NEMATODE ANTIGEN PREPARATIONS

8.1 Introduction

Characterisation studies carried out on *in vivo* and *in vitro* derived mast cells from a variety of species have demonstrated significant differences in their biochemical and functional properties depending on their tissue location or the culture system under analysis (reviewed Galli, 1990; Huntley, 1992). Studies investigating the role of mast cells in nematode infections require access to populations of cells which possess the phenotypic characteristics of MMC found in the ruminant intestine. Techniques which isolate these cells from parasitised intestinal tissues, allow *in vitro* analysis of the types and quantities of mediators released from activated MMC in response to exposure to nematode antigens (Huntley, Wallace and Miller, 1982; Huntley *et al.*, 1984; Huntley, 1991; Jones, Huntley and Emery, 1992).

However, there are a number of practical difficulties associated with their use *ex vivo*. Firstly, it can be hard to achieve sufficiently pure MMC/GL populations in the harvested cells, secondly the cells may be damaged or functionally inactive following the purification procedure (Huntley, 1991) and thirdly, it can be difficult to ensure that the isolated MMC are of a homogenous phenotype. This last point is well illustrated in the rat, where isolation of homogeneous MMC populations from intestinal tissues is difficult due to the co-purification of CTMC present within serosal and submucosal tissues (Befus, 1986; Huntley, 1991).

An alternative approach is to generate mast cells *in vitro* for functional studies, and the previous chapter outlined the optimal conditions for the growth of caprine BMMC using two currently available recombinant ovine cytokines. However, before they can be used to replace isolated cells for functional studies, they must be analysed to ensure that they also possess MMC-associated phenotypic and functional characteristics.

Although the secretogogue responses of sheep MMC have yet to be fully defined, BMMC from sheep generated using LNCM (Haig *et al.*, 1988a) and rOvIL-3

(McInnes *et al.*, 1993; Sture, 1996) provide a convenient source of cells for further studies into the ontogeny and function of ovine mast cells. Functional studies carried out on these cells demonstrate that they can be stimulated to release their granule-associated mediators in the presence of optimal concentrations of calcium ionophore A21387 (Huntley 1991; Huntley *et al.*, 1992a), but are generally refractory to sP or Compound 48/80 (Sture, 1996). As outlined in the general introduction, this response is usually associated with MMC in laboratory animals and humans. Moreover, biochemical investigations, including analyses of proteoglycan content, protease content as well as quantification of other mediators such as acid hydrolases, biogenic amines and leukotrienes have also demonstrated their similarity to isolated ovine MMC (Huntley, 1991; Huntley *et al.*, 1992a).

Phenotypic analyses have yet to be carried out on mast cells derived from goat tissues, and the aim of this chapter will firstly be to isolate MMC and GL from the tissues of animals undergoing natural and experimental parasitic infections. These cells will be analysed for their protease and acid hydrolase content, and attempts will be made to activate them using secretogogues and crude worm antigen preparations. In addition, ultrastructural and biochemical comparisons will be made with goat BMMC generated *in vitro* in the presence of rOvIL-3 and rOvSCF. BMMC will also be analysed for their functional activity in the presence of secretogogues as well as nematode antigen preparations following sensitisation with sera or gastric lymph from animals previously exposed to intestinal nematodes.

Comparisons between these goat and sheep mast cell populations may reveal important differences in their structural and functional characteristics. Moreover, the results should also characterise more fully the phenotype of goat and sheep BMMC cells grown in the presence of rOvIL-3 and rOvSCF. Finally, findings obtained from the worm antigen release studies may indicate the source of allergens important in triggering mast cell-mediated immune responses to nematode parasites.

8.2 Results

8.2.1 Morphological, biochemical and ultrastructural comparisons between isolated goat MMC and BMMC grown in rOvIL-3 and rOvSCF

The isolation procedure was carried out as outlined in Chapter 2.9. Isolations were attempted from six female goats all more than three years of age (Table 8.1). The first three (3352, 3097 and 3021), had been maintained at pasture undergoing natural exposure to parasites. The last three (A62, A26 and Y 55), had been maintained indoors for approximately 6 months prior to the experiment so they were given a mixed infection of 2000 *T. circumcincta* L_3 and 2000 *T. vitrinus* L_3 per os once a week for 8 weeks prior to slaughter (see Chapter 2.2.9), to stimulate an intestinal mastocytosis.

Animal	% MMC + GL in tissue digest	Yield of isolated MMC/GL x 10^6	% MMC + GL in isolated cells
3352	6.5	7.5	69.2
3097	4.75	2	62.5
3021	4.75	5.5	57.5
A62	5.5	3.5	72
A26	5	3	65
Y55	2.5	ND	ND

Table. 8.1 Goat MMC purification results showing the total numbers of cells recovered from discontiuous percoll gradients and the % MMC and GL numbers in the isolated cells as determined by counts on Leishman stained cytosmears. 2.8 x 10^8 dispersed jejunal cells from each animal were layered onto Percoll gradients. ND = not done, no cells recovered from the interface (see text). Cells from A62 and A26 were used for secretogogue release studies.

Leishman stained cytosmears of the purified cells demonstrated the presence of small, basophilic staining, highly granulated cells and larger cells containing less basophilic staining cytoplasmic globules. In both cell types, the granules or globules were so densely packed that they obscured the cell nuclei (Fig. 8.1a). Staining the cytosmears with antibody for GMCP demonstrated that both cell types contained protease (Fig 8.1b), although staining was weaker in the larger cells. In these latter

cells, the staining appeared diffuse within large granules or globules, when compared with the dense granule staining observed in MMC in tissue sections or BMMC (Figs 3.8 a and b and 8.1c). There was also evidence of disruption to the granular structure of the smaller cells in both the Leishman and anti-GMCP stained sections. On the basis of their cytoplasmic morphology, Leishman staining and protease content, the smaller cells were identified as MMC, whilst the larger cells were identified as GL. Contaminating cell types in the purified preparations included erythrocytes (Fig. 8.1a), eosinophilic staining cells, monocytes and a few lymphocytes, which were all negative when stained with anti-GMCP. Total cell recoveries ranged from 2 to 7.5 x 10^6 cells per animal, although in one animal (Y55), there were no cells present at the Percoll interface following centrifugation. The reason for this remains unknown but it may have resulted from aggregation of the cells in the initial tissue digest. In comparison, goat BMMC cultures grown in IMDM, stimulated by the addition of optimal amounts of rOvIL-3 and rOvSCF (Chapter 7) and harvested at day 16, contained up to 80% mast cells as counted on Leishman stained cytosmears (Fig. 8.1c), although only 40-50% of these cells reacted positively with anti-GMCP (Fig. 8.1d). Compared to the isolated MMC, BMMC varied in size and were much less granulated, with some cells containing cytoplasmic vacuoles. Cells with a similar morphology to isolated GLs were not observed in the cultures. Attempts were made to induce GL formation in some BMMC cultures by the repeated addition of secretogogues or worm antigens to cells incubated with or without the addition of serum from parasitised goats, but without success.

Fig. 8.1a Cytosmear of isolated goat MMC and GL stained with Leishman's. Note the highly prominent granules of the MMC and the globules in the larger GL cells (arrows), also the granule disruption in the MMC and the presence of contaminating erythrocytes. 4% Paraformaldehyde (x 500).

Fig. 8.1b Cytosmear of isolated goat MMC and GL stained with polyclonal rabbit anti-GMCP. 4% Paraformaldehyde (x 250).

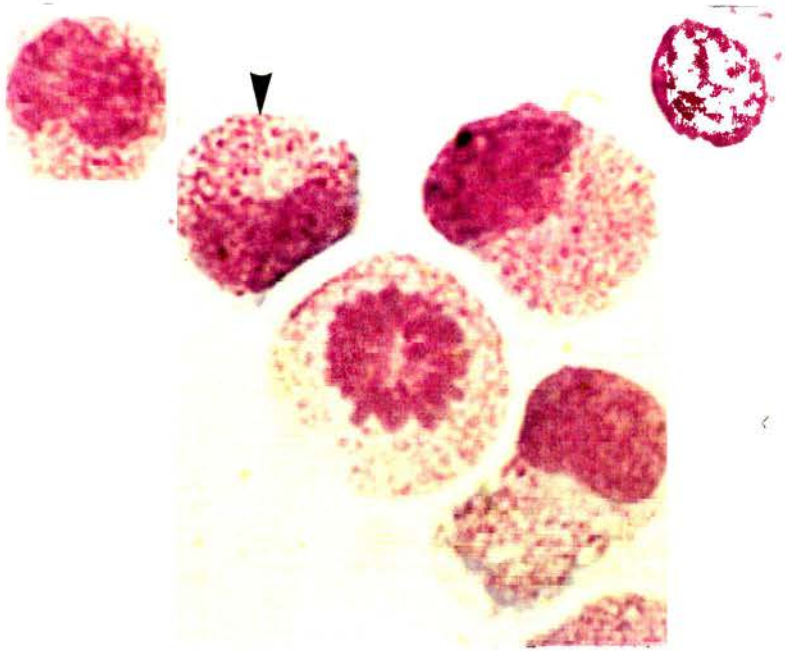


Fig. 8.1c Cytosmear of day 16 goat BMMC generated using rOvIL-3 and rOvSCF stained with Leishman's. Note the immature cytoplasmic granules (arrow). Mitotic figures in the cell in the centre indicate that the mast cell population is still expanding. 4% Paraformaldehyde (x 600).

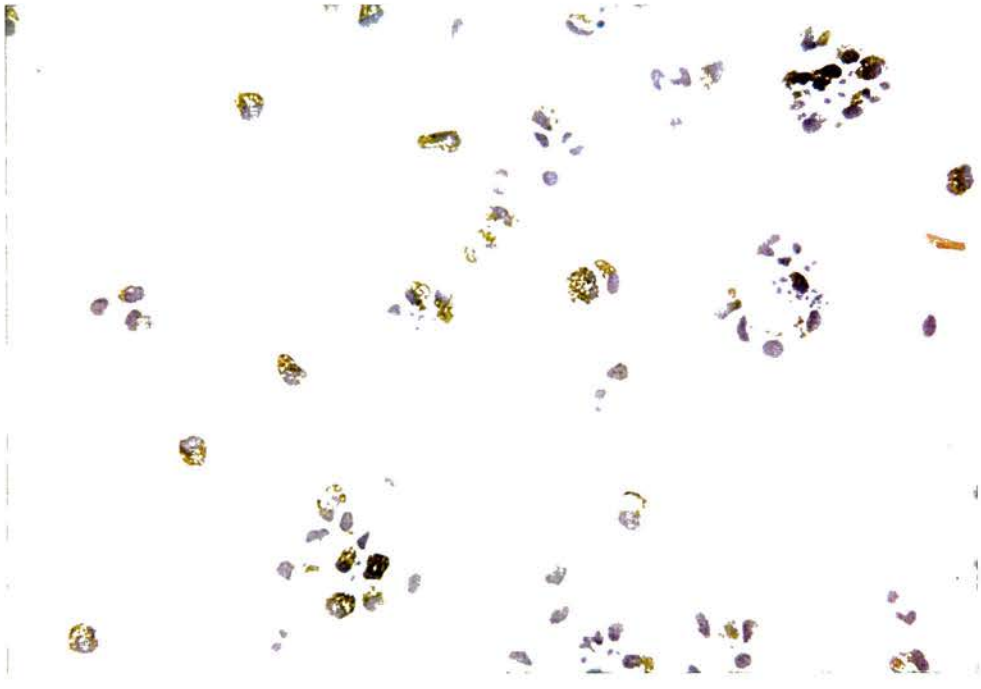


Fig. 8.1d Day 16 goat BMMC generated using rOvIL-3 and rOvSCF stained with polyclonal rabbit anti-GMCP. Note the granular cytoplasmic staining pattern and the proportions of positive staining and negative staining cells. 4% Paraformaldehyde (x 250).

Table 8.2 summarises data for the GMCP, aryl-sulphatase and β-hexosaminidase content in isolated goat jejunal MMC/GL as well as goat and sheep BMMC grown in rOvIL-3 and rOvSCF and harvested on day 16. Corresponding data from Huntley (1991) quantifying the mediator content of sheep BMMC grown in mesenteric LNCM and sheep MMC/GLs recovered from sheep abomasal tissues is also included for comparison.

	Concentration per 10^6 mast cells			
Cells	GMCP/SMCP (ng)	Arylsulphatase (Units)	β-hexosaminidase (Units)	Surface bound Ig
Goat jejunal MMC	84.4 (±49)	74.4 (± 16.3)	0.07 (±0.02)	+
Goat BMMC	5 (±1)	1 (±0.17)	4.9 (±0.51)	+
Sheep BMMC	17.5 (±7.8)	40.2 (±3.2)	2.1 (±0.06)	+
Sheep LNCM-BMMC*	485 (±185)**	43 (±0.4)	0.6 (± 0.03)	+
Sheep abomasal MMC*	3875 (±563)**	88 (±4.7)	0.18 (± 0.08)	+

Table 8.2. Mean (±SEM) comparative mediator content data for goat and sheep mast cells. Cell lysates derived from isolated MMC/GL (MMC) as well as day 16 goat and sheep BMMC grown in rOvIL-3 and rOvSCF or day 21-35 sheep BMMC grown in mediastinal lymph node-derived conditioned medium (LNCM). Results of immuno-fluorescence experiments to detect the presence of surface immunoglobulin are also shown (Surface bound Ig, see below). *= data from Huntley *et al.* 1992. **= lysates tested with SMCP ELISA, all other samples were tested with the cross-reactive GMCP ELISA (Chapter 3). Results for goat jejunal MMCs are the mean of five cell isolation experiments (see Table 8.1). Results for the BMMC represent the mean of at least 4 cell preparations from 2 separate cultures.

The results show that the isolated goat MMC/GL contained significantly more GMCP than goat and sheep BMMC grown using recombinant cytokines ($P < 0.01$). However, they also contain less protease than sheep BMMC grown in LNCM, whilst a particularly striking finding was the substantial difference in the protease content of goat MMC (84 ng/10^6 mast cells) when compared to sheep MMC (3875 ng/10^6 mast cells). Sheep BMMC grown using recombinant cytokines were also found to contain significantly less ($p < 0.001$) SMCP than either the equivalent sheep BMMC stimulated using LNCM or the sheep abomasal MMC/GL preparations. In contrast to their protease content, no notable differences in arylsulphatase content were observed in the isolated MMC from both species or sheep BMMC grown in LNCM or recombinant ovine cytokines. However, the isolated MMC did contain more arylsulphatase when compared with the sheep BMMC cells. In accordance with the findings in the previous chapter, goat BMMC were found to be particularly deficient in aryl-sulphatase, containing significantly less ($p < 0.0001$) than the other cells. As noted in earlier studies on sheep BMMC (Huntley, 1992 and Sture, 1996), *in vitro* derived BMMC from both species were found to contain significantly more β-hexosaminidase than the isolated goat or sheep MMC ($p < 0.001$). In addition, the goat and sheep BMMC grown using recombinant cytokines also contained more β-hexosaminidase than the LNCM stimulated sheep BMMC cultures.

A morphological assessment of cytoplasmic granule formation was made by transmission electron microscopy (TEM). Figures 8.2a and b shows micrographs of day 16 goat and sheep BMMC grown using rOvIL-3 and rOvSCF. Figure 8.2c shows a fully granulated, isolated goat MMC, whilst figures 8.2d and e show a degranulating isolated goat MMC and an isolated goat GL respectively. The goat and sheep BMMC (Fig. 8.2a and b) contain much less electron dense material within their cytoplasmic granules when compared with those in the isolated MMCs (Fig. 8.2c and d). The granules of the BMMC also appear incomplete and poorly formed, containing

material that is more reminiscent of the floccular matter prominent within the coalescing cytoplasmic structures of the GL (Fig. 8.2e) than the homogeneous opacity of the isolated MMC granules. Sheep BMMC also contain marginally more electron dense material within their cytoplasmic structures than goat BMMC, which may correlate with the greater amounts of mast cell protease and aryl-sulphatase found within these cells (Table 8.2). The degranulating MMC (Fig. 8.2d) was probably damaged during the purification process, and may not be representative of a mast cell undergoing activation by normal physiological mechanisms. Nevertheless, the cytoplasmic granules exhibit some of the characteristics previously reported in human nasal and lung mast cells undergoing FcεRI mediated activation. These include a reduction in individual granule size with relocation to the periphery of the cytoplasm and loss of their homogeneous appearance to form intense staining rope-like structures which typically precedes the particulate appearance of fully discharged granules (Freidman and Kaliner, 1981). However, possibly due to species differences, the scroll and lattice-like structures often observed in human mast cells cytoplasmic granules (reviewed Dvorak, 1986) were not observed. The GL in Figure 8.2e exhibits the large, coalescing cytoplasmic globules that dominate the cell and obscure the nucleus in Leishman stained cytosmears (Fig. 8.1a), although there are still one or two areas of electron-dense material, indicating that the cells have not discharged completely.

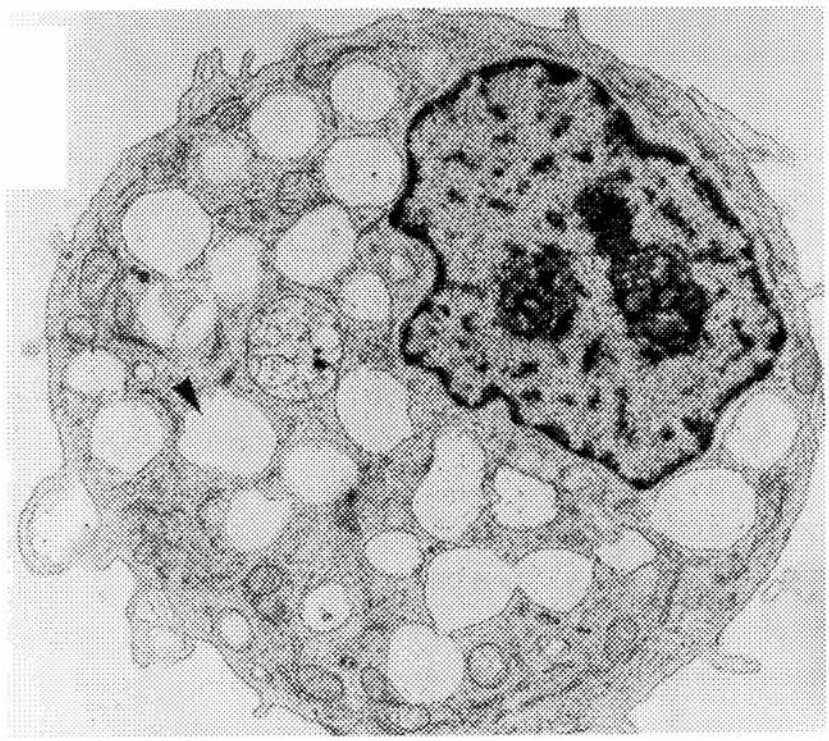


Fig 8.2a Day 16 goat BMMC generated in rOvIL-3 and rOvSCF, note the lack of electron dense material in the granules (arrow) (x10 000). Cell pellet fixed in 3% gluteraldehyde and 0.1M sodium cacodylate for 24 hours at 4 ^{o}C.

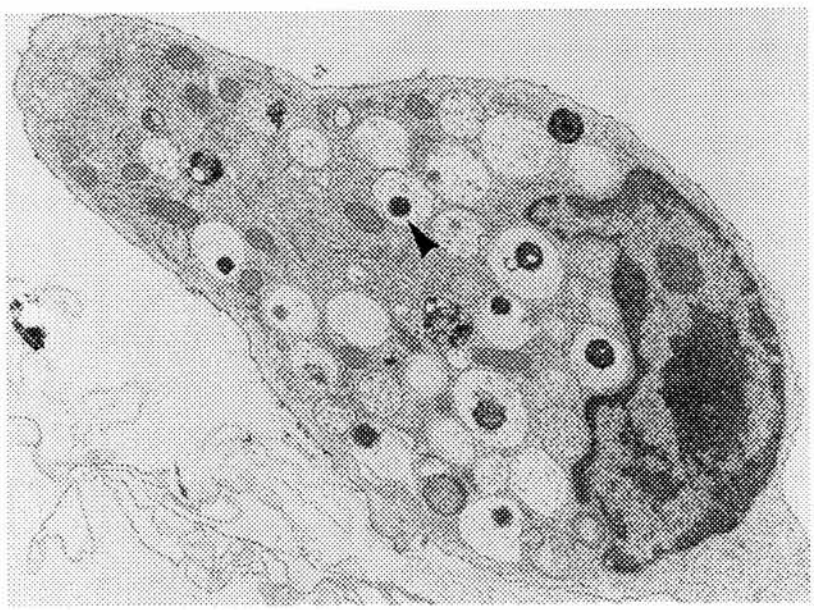


Fig. 8.2b Day 16 sheep BMMC generated in rOvIL-3 and rOvSCF, note the presence of some electron dense material in the cytoplasmic granules (arrow) (x10 000). Cell pellet fixed in 3% gluteraldehyde and 0.1M sodium cacodylate for 24 hours at 4 ^{o}C.

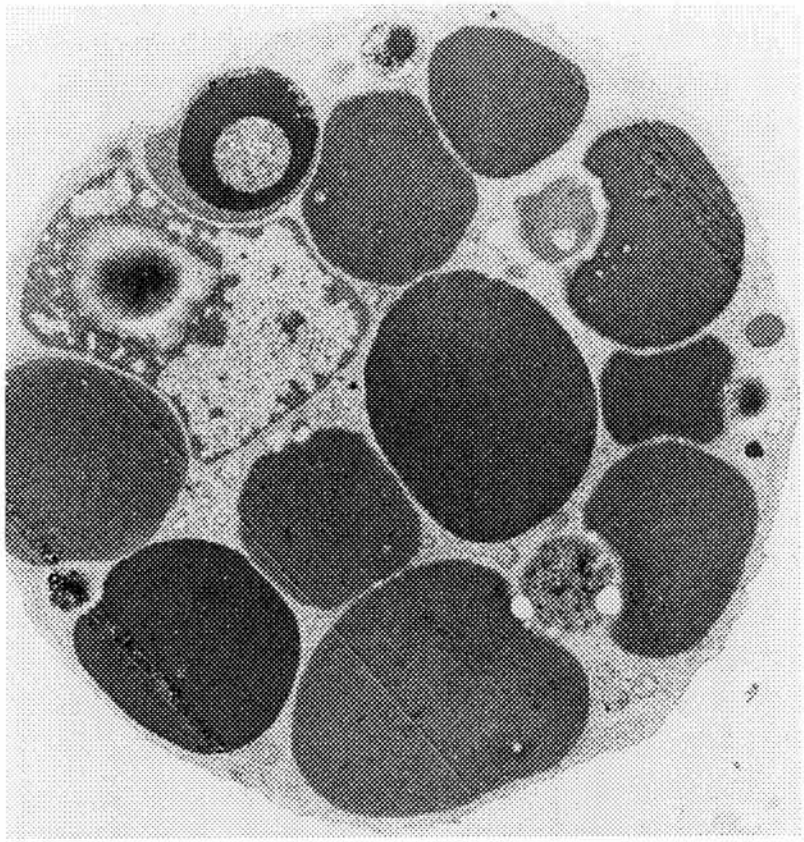


Fig. 8.2c Fully granulated isolated goat MMC (x10 000). Cell pellet fixed in 3% gluteraldehyde and 0.1M sodium cacodylate for 24 hours at 4 ^{o}C.

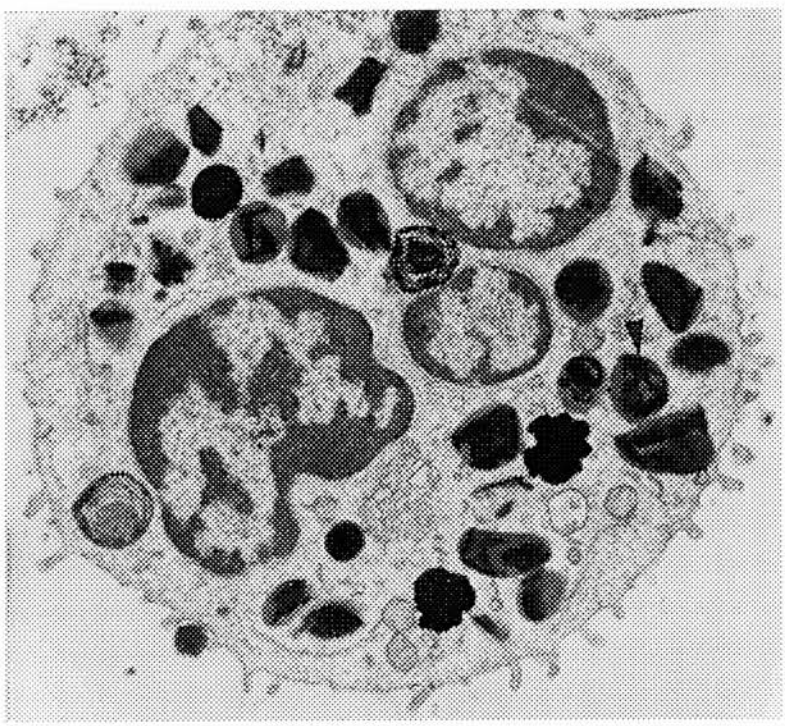


Fig. 8.2d Degranulating isolated goat MMC, note non-homogeneous 'rope-like' densities in the discharging granules (arrow) (x10 000) Cell pellet fixed in 3% gluteraldehyde and 0.1M sodium cacodylate for 24 hours at 4 ^{o}C.

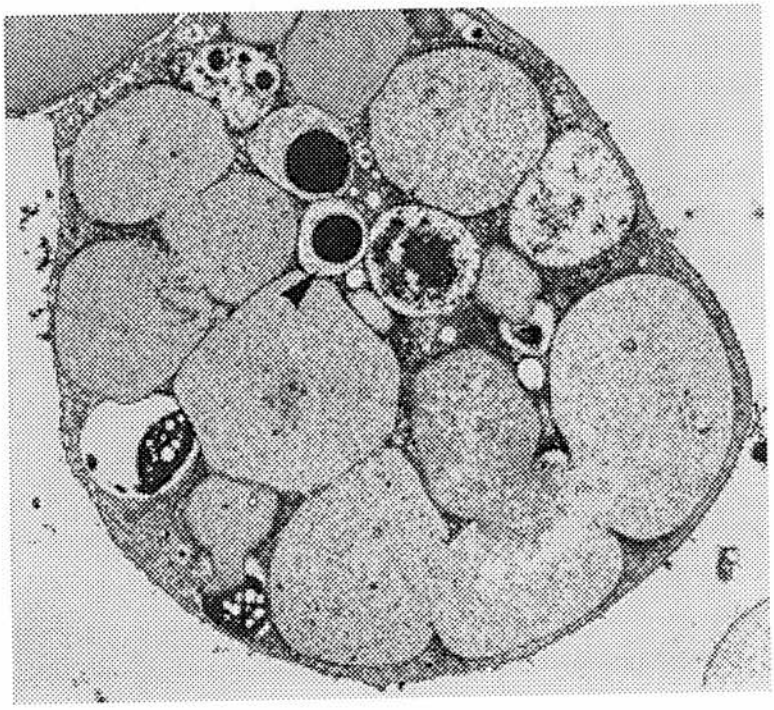


Fig. 8.2e Isolated goat GL, note remaining electron dense granules (arrow) (x 3550). Cell pellet fixed in 3% gluteraldehyde and 0.1M sodium cacodylate for 24 hours at 4 ^{o}C.

8.2.2 Demonstration of immunoglobulin binding on the surface of goat MMC and goat BMMC

This was carried out as described in Chapter 2.11.3 using IgE positive serum samples obtained from the animals used in the experiment described in Chapter 6. Controls consisted of BMMC cells incubated in IgE positive serum that had been heat inactivated at 56 ^{o}C for 2 hours, MMC, or IgE positive serum-sensitised BMMC that had been incubated with an anti-border disease virus monoclonal antibody (VPM-12) in place of the anti-ovine light chain antibody (VPM-8). Figures 8.3 a to d show immunolocalisation confined to the surface of MMC (Fig. 8.3a) and the IgE positive serum incubated BMMC cells (Fig. 8.3c), but little reactivity was observed in preparations of MMC incubated with VPM-12 (Fig. 8.3b) or BMMC incubated with heat-inactivated serum (Fig. 8.3d) (BMMC incubated with IgE positive serum and VPM-12 were also negative, not shown). The experiment was repeated for pre-sensitised sheep BMMC generated in rOvIL-3 and rOvSCF, which also exhibited surface localisation of reaction product (not shown). The findings are in accordance with those of Huntley (1991), who demonstrated surface bound immunoglobulins on LNCM stimulated sheep BMMC and isolated sheep MMC (Table 8.2).

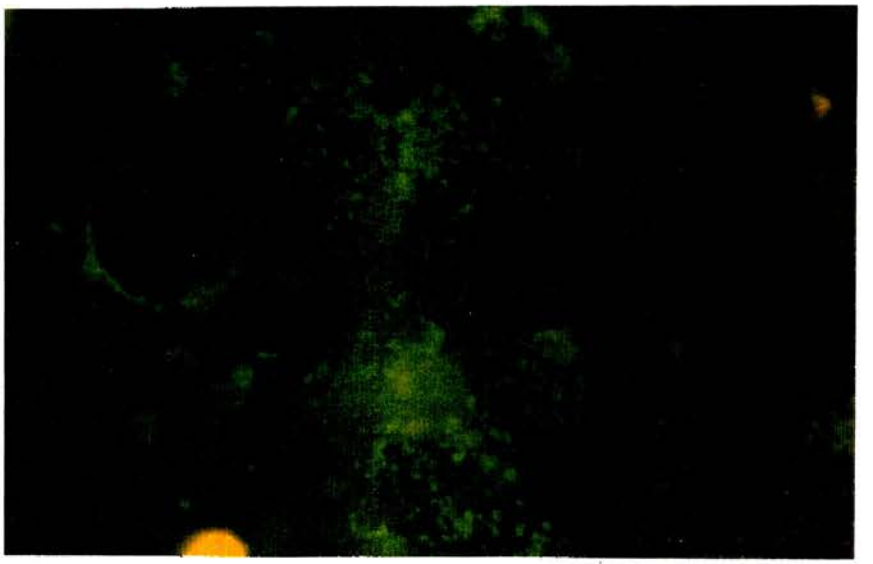


Fig. 8.3a Goat MMC/GL incubated with mouse anti-ovine light chain monoclonal antibody (VPM-8) and anti-mouse IgG FITC. (x 400)

Fig. 8.3b Goat MMC/GL incubated with an irrelevant mouse anti-border disease virus monoclonal antibody (VPM-12) and anti-mouse IgG FITC. (x 400)

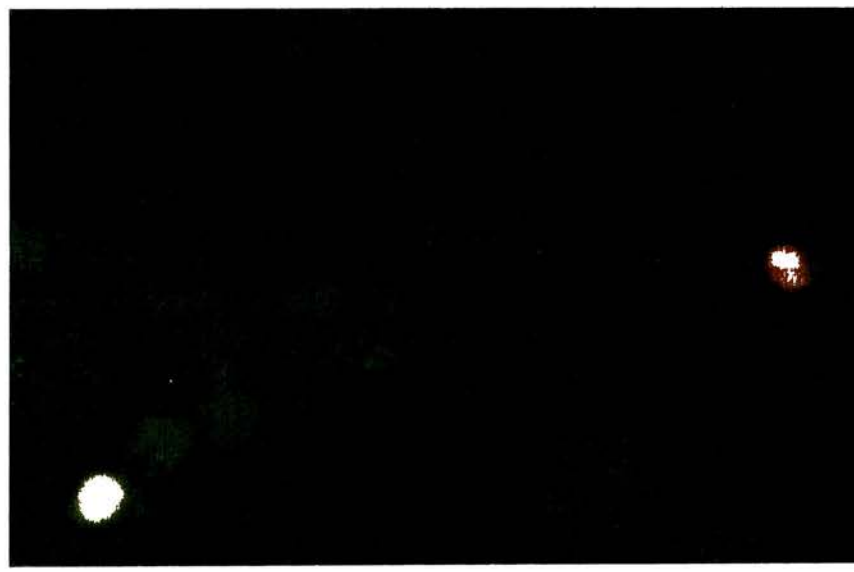


Fig. 8.3c IgE positive serum-sensitised day 16 goat BMMC grown in rOvIL-3 and rOvSCF incubated with VPM-8 and anti-mouse IgG FITC. (x 400)

Fig. 8.3d Heat inactivated IgE positive serum-sensitised day 16 goat BMMC grown in rOvIL-3 and rOvSCF incubated with VPM-8 and anti-mouse IgG FITC (x 400)

8.2.3 Functional characterisation of goat BMMC and MMC: Their responses to secretogogues.

Duplicate 0.2 - 2 x10^6 aliquots of MMC or BMMC were incubated with 10^{-4} to 10^{-7}M calcium ionophore A21387, 10^{-2} to 10^{-4}M sP or 250µg/ml Compound 48/80 for 45 minutes at 37 ^{o}C (Sture, 1996). After centrifugation, the resultant cell pellets along with the supernatants were collected and tested for their GMCP, aryl-sulphatase and β-hexosaminidase content as outlined in Chapter 2.12. Paired controls incubated in ionophore diluent or Earles + 2% FCS only were also tested for positive activation or background spontaneous release respectively. The final background corrected results for mediator release into the supernatant were then expressed as a percentage of the total cell mediator content detected in equivalent cell lysate preparations as described in Chapter 2.12.6.

Duplicate aliquots of freshly purified MMC from 2 animals (A26 and A62) were initially stimulated with secretogues as outlined above. However, supernatants from the diluent only controls demonstrated very high spontaneous release of GMCP (56.7 ± 28.1%), aryl-sulphatase (56.4 ± 19.8%) and β-hexosaminidase (56.6 ± 21.2%) which was higher than the secretogogue stimulated samples or diluent controls. Due to time constraints and difficulties associated with obtaining further supplies of isolated mast cells, these experiments were discontinued and the remaining functional characterisation work was performed on goat BMMC generated *in vitro.*

The secretogogue-induced GMCP, β-hexosaminidase and aryl-sulphatase release release results for day 16 to 22 goat BMMC grown in rOvIL-3 and rOvSCF are shown in Table 8.3. They demonstrate a significant ($p< 0.05$) dose response effect, with maximal release demonstrated when the cells are stimulated with 10^{-6}M calcium ionophore (mean 70 to 80% release for β-hexosaminidase and aryl-sulphatase and 39.3% for GMCP). The cells were relatively refractory to the effects of the other secretogogues, although compound 48/80 and 10^{-4}M sP stimulated the cells to release 23.5 ± 2.1% and 8.2 ± 4.46% of their GMCP content respectively.

Secretogogue	GMCP n = 3	Aryl-sulphatase n = 2***	β-hexosaminidase n = 3
A21387 10^{-4}M	12.1 (±4.7)	19.2 (±4.4)	11.3 (±2.6)
A21387 10^{-5}M	24.3 (±7.3)	18.2 (±0.6)	23.4 (±9.8)
A21387 10^{-6}M	39.3 (±6.35)	70.2 (±11.8)	76.6 (±3.4)
A21387 10^{-7}M	21.7 (±5.9)	25.5 (±5.3)	12.5 (±14.2)
*Diluent + ethanol release	14.5 (±1.1)	18.6 (±4.4)	16.7 (±2.3)
Compound 48/80 (250 μg/ml)	23.5 (±2.1)	0	2.3 (±2.1)
Substance P 10^{-2}M	3.5 (±4.5)	0	0.7 (±0.8)
Substance P 10^{-4}M	8.2 (±4.5)	0	0
**Diluent only	8.1 (±6.7)	0.35 (±0.3)	10.5 (±3.8)

Table 8.3. Mean (±SD) percentage mast cell GMCP, aryl-sulphatase and β-hexosaminidase release after 45 minutes incubation with secretogogues using day 16 to day 22 goat BMMC fed with 10^{-2} rOvIL-3 and 10^{-2} rOvSCF. All tests were carried out on duplicate samples in three experiments, each experiment used cells taken from separate time response cultures. *Cells incubated in diluent containing the equivalent concentration of ethanol required to dissolve 10^{-4}M A21387 (see 2.11.1). A21387 results are corrected for this spontaneous release. **Cells incubated in diluent only. Compound 48/80 and substanceP results are corrected for this spontaneous release. ***Aryl-sulphatase tested in two experiments only due to the low levels of this enzyme found in day 16 goat BMMC. Mediator release results from individual experiments shown in Appendix B (pp iii - v).

8.2.4 Functional activation of goat and sheep BMMC: Their response to stimulation with crude nematode antigen preparations.

Results from the secretogogue release experiments indicated that the BMMC cultures possess a functional phenotype similar to isolated MMCs characterised previously from other species (reviewed Barrett and Pearce, 1993). An experiment was therefore set up to examine the cell responses to stimulation with crude nematode antigen preparations from *T. circumcincta* and *H. contortus* L_3 and L_5/adults. These preparations were kindly donated by Dr. D. Knox and Dr. J. Huntley having been prepared as outlined in 2.11.4 using larvae and adults cultured or collected by Dr. F. Jackson. Freshly thawed aliquots of antigen were diluted to 100µg/ml in Earles medium without FCS immediately prior to use, to minimise proteolytic damage to potential antigens within the extracts.

Aliquots of day 16-22 goat and sheep BMMC containing 0.5 - 2 x 10^6 cells were pre-incubated in IgE-positive goat serum. In addition, goat BMMC were also pre-incubated with abomasal afferent lymph collected from an ADCM infected adult doe that had been cannulated (Smith , Jackson, Jackson and, Williams, 1983a; Patterson, 1996) and challenged with 50, 000 *T. circumcincta* L_3 10 days before sampling. The lymph sample, kindly provided by Dr. D. M. Patterson, contained 2.52 µg/ml total IgE by dot blot assay. Negative control samples consisted of cells pre-incubated with serum that had been heat inactivated at 56 ^oC for 2 hours. Unfortunately, due to limitations in the amounts of available lymph, it was not possible to sensitise the sheep BMMC or incubate equivalent negative controls for the lymph sensitised goat BMMC cells. Following sensitisation, the cells were washed and incubated as described in 2.11.5 with 50µg/ml of *T. circumcincta* L_3 excretory/secretory antigens (*T.c.*L_3ES), *T. circumcincta* L_3 whole worm homogenate antigens (T.c.L_3WWA), *T. circumcincta* L_5 excretory/secretory antigen (T.c.L_5ES), *T. circumcincta* L_5 whole worm homogenate antigens (T.c. L_5 WWA),

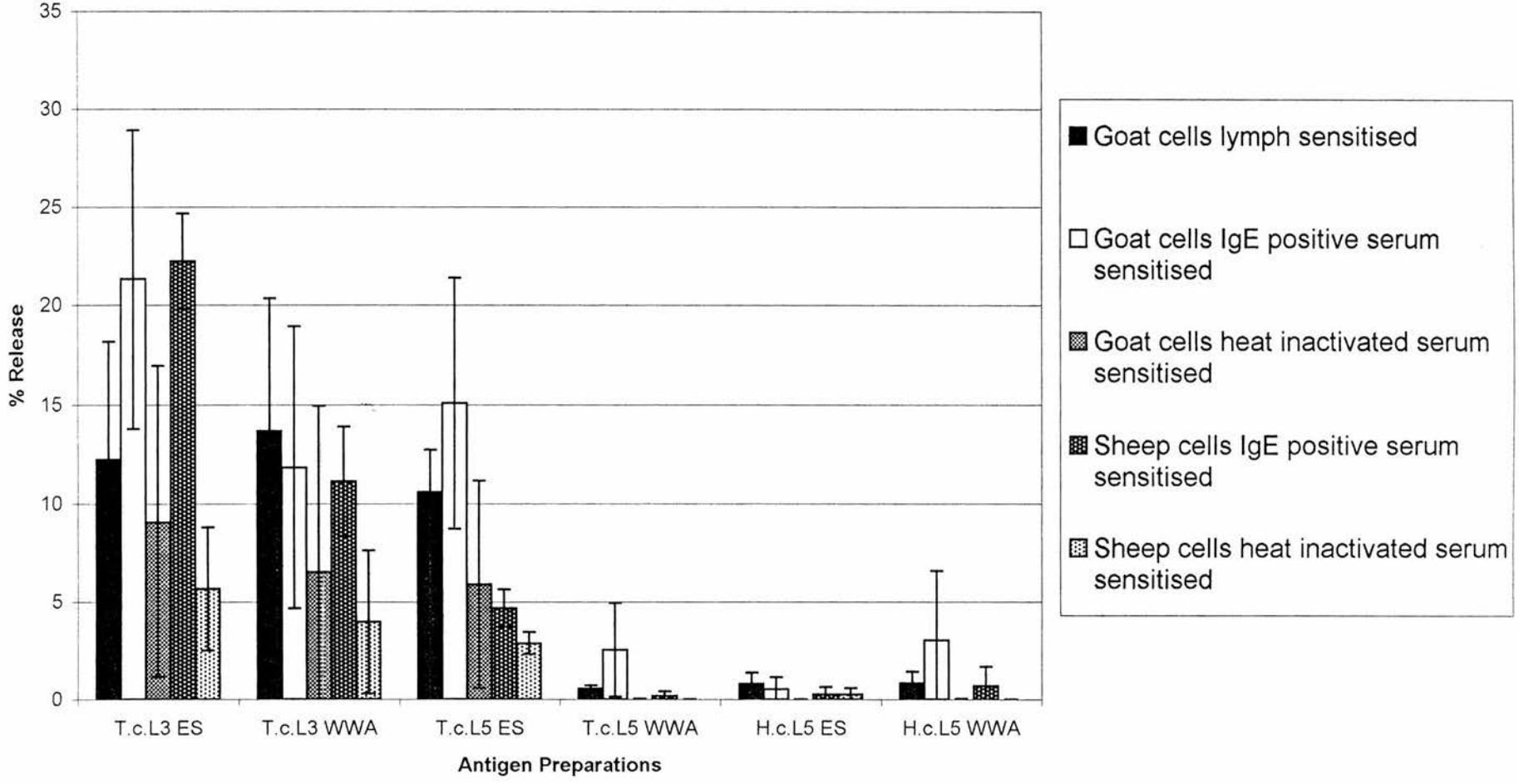
H. contortus L_5 excretory/secretory antigens (H.c. L_5ES) or *H. contortus* L_5 whole worm homogenate antigens (H.c. L_5 WWA). After incubation, the cell pellets and supernatants were harvested and tested for their GMCP, β-hexosaminidase and aryl-sulphatase content. Aryl-sulphatase results could not be obtained for cells incubated with T.c. L_3 WWA due to the presence of high levels of innate aryl-sulphatase activity in the antigen preparation which interfered with the assay (Fig. 8.4c). Duplicate samples of the goat BMMC were tested in three experiments whilst duplicate samples of sheep BMMC were tested in two experiments. Each experiment involved using cells from different time course cultures. The results were corrected for spontaneous release from cells undergoing the sensitisation step only. The mean (± SD) percentage mediator release results for β-hexosaminidase, GMCP and aryl-sulphatase release are summarised in Figure 8.4a, b and c respectively.

The data shows that although there was considerable variation in individual results, mean percentage release was generally highest for goat and sheep cells pre-sensitised with IgE positive serum or lymph and incubated with the *T. circumcincta* antigen preparations. This resulted in the cells releasing approximately 10 to 40% of their mediator content although the highest mean percentage release results (60.2 ± 14.6% and 68.5 ± 11.73% for lymph and serum sensitised goat BMMC and 50.3 ± 42.1% for the serum- sensitised sheep BMMC), were obtained for GMCP release in the presence of T.c. L_3WWA. Despite the mean percentage release results being invariably greater for sensitised cells, none of the results were significantly higher ($p > 0.05$) than those obtained for the corresponding heat inactivated serum-sensitised control cells. The mean percentage release results obtained for the heat inactivated serum-sensitised control cells were also higher than expected although the overall pattern of release with each antigen preparation was similar to that seen in non-heated serum and lymph-sensitised cells. A further consistent feature, was the apparently selective release of GMCP and aryl-sulphatase from sensitised and control

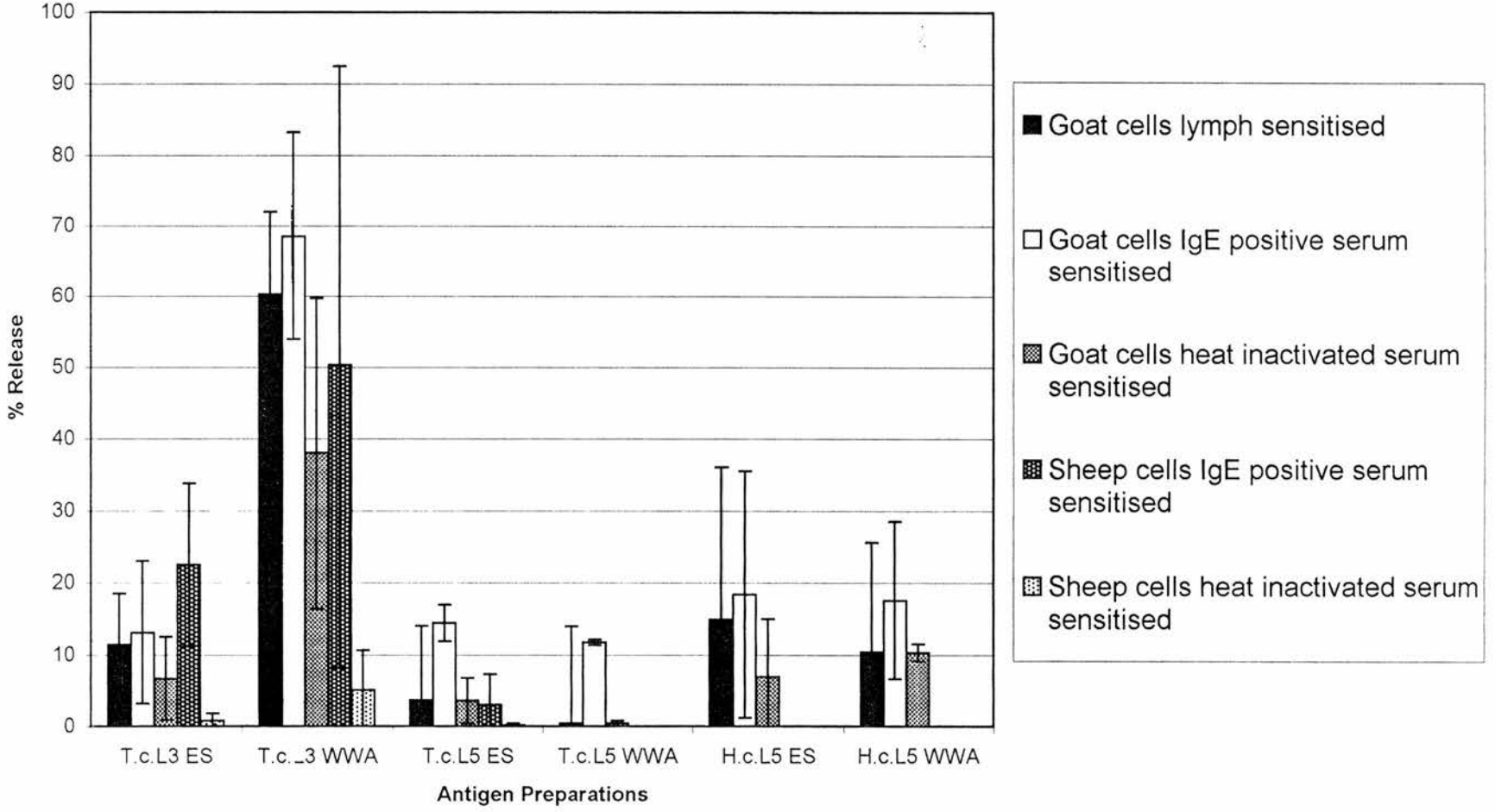
goat cells stimulated with the *T.c.*L_5 and *H.c.*L_5 worm antigen preparations (Fig.8.4 b and c). This effect was also seen to a lesser extent for β-hexosaminidase release from goat cells stimulated with T.c.L_5 ES (Fig.8.4a).

Fig. 8.4 a,b and c (following pages). Mean (±SD) % release of β-hexosaminidase a), GMCP b) and aryl-sulphatase c) from day 16-22 goat and sheep BMMC grown in rOvIL-3 and rOvSCF. Cells sensitised with goat serum (IgE-positive serum) or lymph from animals immunised with *T. circumcincta* or serum from the same animals that had been heated to 56 ^{o}C for 2 hours (heat inactivated serum). Cell release stimulated with crude excretory/secretory (ES) and whole worm homogenate (WWA) larval antigen preparations: T.c.L3 = *T. circumcincta* L_3, T.c.L5 = *T. circumcincta* L_5. H.c.L5 = *H. contortus* L_5. (Aryl-sulphatase release from cells stimulated with T.c.L3WWA not shown due to interference with the assay by the worm antigen preparation.). Results shown for the goat BMMC are the mean of 3 separate experiments carried out in duplicate samples. Results shown for the sheep BMMC are the mean of 2 separate experiments carried out on duplicate samples. Individual mediator release results for each experiment are shown in appendix B (pp vi - viii).

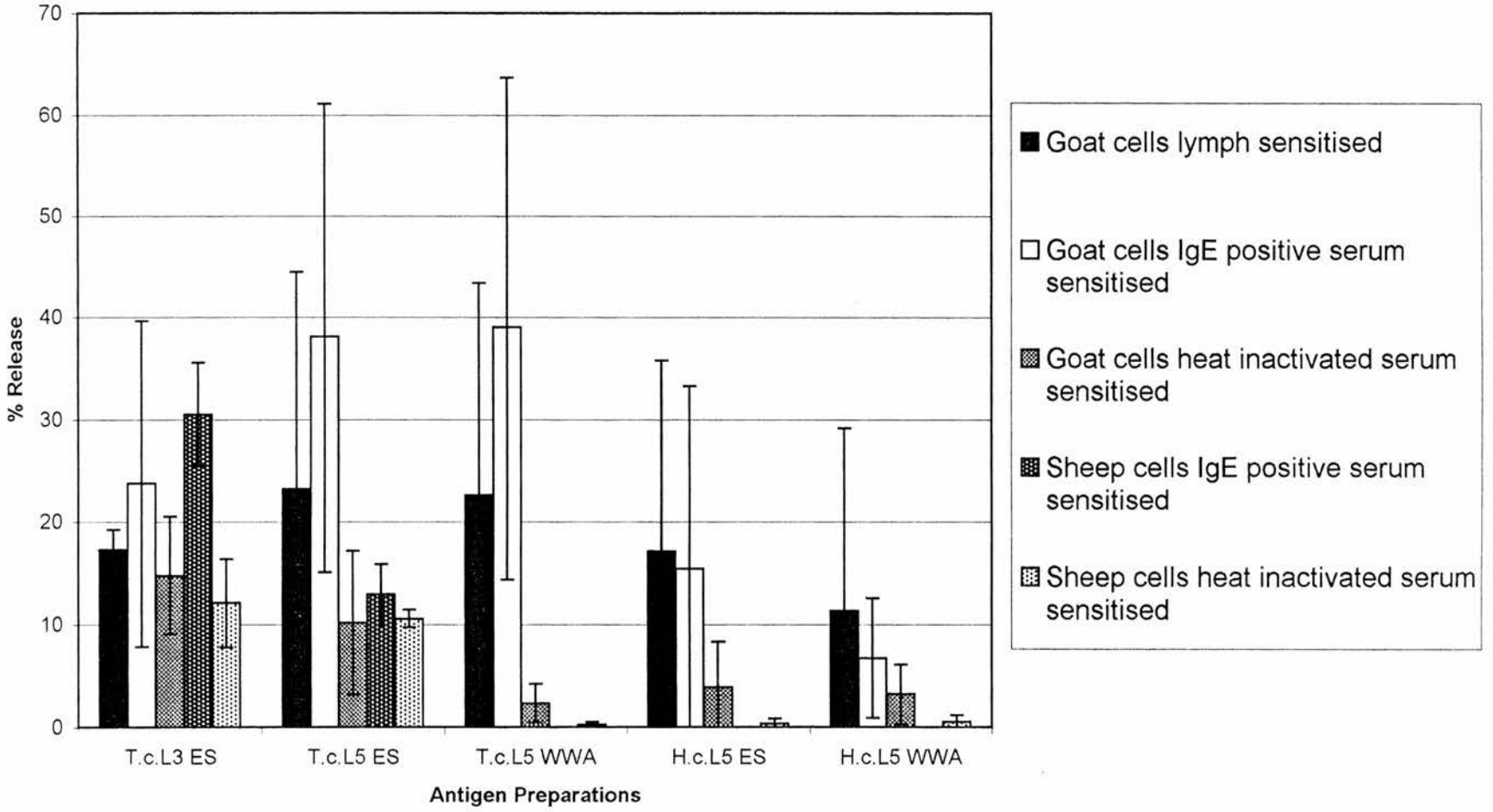
a) % Beta-Hexosaminidase Release from Goat and Sheep BMMC



b) % GMCP release from Goat and Sheep BMMC



c) % Aryl-Sulphatase Release from Goat and Sheep BMMC



8.3 Discussion

The results outlined in this chapter indicate that there are some similarities in the structure, biochemistry and function of isolated goat MMC and goat BMMC grown using recombinant ovine cytokines. In terms of morphology, Leishman's stain reveals the presence of numerous granules in both cell types and, although the BMMC granules are much smaller and less dense than the MMC granules, GMCP can be localised to them by immunohistochemical staining. In addition, weak staining for GMCP can also be localised to the large cytoplasmic globules of GL that are co-purified with isolated MMC, further indicating that the relationship between the two cell types is similar to that demonstrated previously in sheep (Huntley *et al.*, 1984).

The granule constituents of both cell populations are also similar with both cell types containing GMCP, aryl-sulphatase and β-hexosaminidase. The quantities of particularly GMCP and aryl-sulphatase are significantly lower in the goat BMMC when compared to the isolated goat MMC/GL. Isolated goat MMC/GLs also contained approximately 45 times less GMCP per cell but similar quantities of aryl-sulphatase and β-hexoaminidase when compared with values obtained for SMCP in isolated sheep abomasal MMC (Huntley, 1991). Interestingly, this difference in protease concentrations is of a similar magnitude to that found for adult sheep abomasal and goat jejunal tissues (Huntley *et al.*, 1995). However, it should also be noted that there was morphological and ultrastructural evidence of granule disruption in the isolated goat MMC (Fig 8.1a and b; Fig. 8.2d) and that they demonstrated a high degree of spontaneous release in attempted functional characterisation studies. As a consequence, the mediator results obtained from these samples may be artificially low due to cell damage and/or degranulation during the purification procedure.

Ultrastructurally, the granules in goat BMMC appear poorly formed when compared with those in isolated MMC. In particular, the scarcity of electron-dense material (fig 8.2a) which is indicative of proteoglycan granule matrix bound protease and histamine (Dvorak, 1986), agrees with the lack of protease found in BMMCs by

ELISA. Moreover, the presence of particulate material within the BMMC and GL suggests the presence of either incompletely formed granules in the BMMC which may represent cellular immaturity, or in the GL, granules which have already discharged, leaving the residual material seen within their cytoplasmic globules. The apparent relationship between goat MMC and GL is also strengthened by the presence of some electron-dense granules adjacent to the cytoplasmic globules of the GL which parallels the pattern of anti-GMCP staining often seen in GLs in tissues *in vivo* (chapter 3, Fig. 3.8; a and b).

Sheep BMMC generated in LNCM contained significantly more protease than either goat or sheep BMMC generated using recombinant ovine cytokines (Table 8.2). In addition to SMCP, Huntley (1991) also found evidence of a heavier antigenically-related 31.5 kD [^{3}H]- di-isopropyl fluorophosphate (DFP) binding serine-protease band which appeared by day 7 in LNCM stimulated sheep BMMC cultures. This band was not detected in equivalent samples of isolated sheep abomasal MMC. For safety reasons, [^{3}H]-DFP binding analyses were not carried out on goat MMC or BMMC samples, although Western blots of Day 16 goat BMMC cell extracts probed with anti-GMCP failed to detect the presence of an extra band (see chapter 3, Fig. 3.5 lane 3). This failure may be due to lack of protein expression or lack of sensitivity in the blotting procedure. Alternatively, post translational modifications such as glycosylation, to a single parent protease may occur in sheep BMMC, but not goat BMMC, producing the different electrophoretic mobilities shown by the two bands. There is evidence that glycosylation is a feature of some samples of SMCP isolated from abomasal tissues and possibly immunoaffinity-purified GMCP (A. Pemberton, personal communication, Chapter 3, Fig. 3.3b) , whilst at least 2 potential glycosylation sites were detected on cDNA sequences obtained for GMCP/SMCP I and II (chapter 5). However, glycosylation of GMCP present in goat MMC or BMMC remains to be demonstrated and would require experiments involving incubation of GMCP samples with glycosidase enzymes. It should be noted that sheep BMMC cultures produced by Huntley (1991) were grown using LNCM and not rOvIL-3 and

rOvSCF which may have resulted in these cells being exposed to additional cytokines or growth factors responsible for controlling protease expression. It is therefore also possible that this extra protease represents SMCP II (see Chapter 5) although further biochemical characterisation studies including NH_2-terminal sequencing would be required to confirm this. In any case, the the significantly higher concentrations of SMCP detected in the LNCM stimulated sheep BMMC may have been due, at least in part, to increased expression of the additional cross-reactive 31.5 kD protease.

Attempts to characterise isolated goat MMC in terms of their functional response to calcium ionophore A21387, sP and compound 48/80 were unsuccessful due to excessive spontaneous release from unstimulated control samples. It is likely that the cells were damaged during the isolation process, since histochemical and ultrastructural observations indicated extensive disruption to the arrangement of their cytoplasmic granules (Fig. 8.1 a and b; Fig. 8.2d). Similar problems were encountered previously with isolated sheep MMC (Huntley, 1991) although since then, improvements in the technique have enabled functionally viable cells to be isolated from this species (Jones *et al.*, 1992; Bendixsen *et al.*, 1995).

In view of this failure to functionally characterise isolated goat MMC, it was not possible to categorise goat BMMC as being 'MMC-like' in their response to ionophores. However, the majority of characterisation studies on BMMC generated *in vitro* using LNCM, IL-3 and SCF carried out in rats, mice, humans and sheep have produced cultures where the majority of cells are biochemically and functionally similar to MMCs. (Haig *et al*, 1982; Shanahan, Lee, Denburg, Bienenstock and Befus, 1986; McMenamin, Haig, Gibson, Newlands and Miller, 1987; Haig *et al.*, 1988a; Broide, Metcalfe and Wasserman, 1988; Macdonald et. al., 1989; Sredni, Friedman, Bland and Metcalfe, 1983; Gilead, Rahamin, Ziv, Or and Razin, 1988; Huntley *et al.*, 1992).

In the present study, the greatest release from goat BMMC occurred with 10^{-6}M A21387. This was in agreement with earlier studies using sheep BMMC grown in LNCM (Huntley, 1991, Huntley *et al.*, 1992). However, the goat BMMC

also demonstrated approximately 25% and 10% GMCP release in response to stimulation with Compound 48/80 and sP respectively, which has been associated with the activation of mast cells with a CTMC phenotype (Barrett and Pearce, 1993). This moderate response to CTMC-associated secretogogues has been demonstrated in mouse, and more recently, sheep BMMC generated with IL-3 (Chiu and Burrall, 1990; Sture, 1996) and suggests that they may either be atypical in their functional activity, or that there is phenotypic heterogeneity within the cultured BMMC populations.

Since the majority of goat BMMCs produced functional responses in keeping with an MMC phenotype, it was considered that experiments to examine the functional responses of these cells following exposure to nematode antigens were justified. The results demonstrated a high degree of variability in the BMMC responses from both species, although there was a trend towards enhanced release from cells pre-sensitised with serum or lymph and challenged using *T. circumcincta* antigen preparations. This may indicate that an antibody-mediated release mechanism involving surface bound *T. circumcincta* specific IgE was involved, since the BMMC were pre-sensitised with serum or lymph, containing elevated total IgE levels, from animals undergoing active infection with *T. circumcincta* (chapter 5; Patterson, 1996). However, cells pre-incubated with heat inactivated serum also released notable quantities of mediators. This effect was demonstrated in both goat and sheep BMMC incubated with *T. circumcincta* antigens and in goat BMMC incubated with *H. contortus* antigens. The results were all corrected for spontaneous release, which represented the basal secretion during incubation, indicating that release occurred in these control samples following addition of the nematode antigen preparations. This may have been mediated as a result of incomplete inactivation of serum IgE, despite heat treatment, or it may also indicate the presence of other antibody-dependent release mechanisms that do not involve IgE but perhaps IgM or IgG. In this respect, the release of acid hydrolases, PGD_2 leukotrienes following cross linking of IgG bound to Fcγ receptors on the surface of murine BMMC has been previously reported

(Katz *et al.* 1992). Although, if this was the case in the current experiments, then it occurred despite the fact that heat inactivated serum sensitised control cells did not exhibit immunofluorescence for surface immunoglobulin (Fig 8.3 d). Another possible explanation would be the presence of an additional, and as yet undefined, antigen dependent but non-antibody mediated release mechanism operating in both the IgE positive and heat inactivated control sensitised samples.

Overall, these findings demonstrate that worm antigen preparations may be able to stimulate mediator release from goat and sheep BMMC *in vitro* and, although the results from this experiment are not significant due to large variations between samples, mean release is possibly enhanced by pre-incubating the cells with IgE-containing serum or lymph. The ability of the different L_3 and L_5 stages of *T. circumcincta* at stimulating BMMC degranulation also suggests that the response may not be confined to stage-specific larval antigens, although greatest release was usually obtained from cells incubated with antigens from L_3 preparations.

CHAPTER 9

GENERAL DISCUSSION

9.1 General Discussion

The work described in this thesis has contributed to studies on the comparative roles played by MMC and GL in gastro-intestinal nematode infections of small ruminants. By using the goat as an experimental model, insights have been gained into MMC and GL responses which may be involved in host regulation of gastro-intestinal nematode burdens. Moreover, confirmation of the relative inability of goats to regulate their worm burdens when compared with sheep (Huntley, 1995; Chapter 6) provides ample opportunities for future comparative studies investigating other aspects of the ruminant mucosal-immune response to nematodes.

In terms of caprine MMC responses, the most notable achievement was the purification of low amounts of a neutral granule protease (GMCP) from parasitised goat jejunal tissues (Chapter 3) which, after histochemical and immunological analysis, was localised to goat gastro-intestinal MMC and GL. After further biochemical and molecular analyses, this enzyme was also shown to possess similar characteristics to the more abundant equivalent sheep mast cell enzyme, SMCP. The development of an ELISA for GMCP which was cross-reactive for SMCP validated the previous use of SMCP antibodies for the measurement of GMCP (Huntley *et al*, 1995), and allowed subsequent measurements of comparative mast cell chymase concentrations in tissues and cell supernatants to be made with confidence.

The production of antibodies to GMCP also enabled the most detailed examination of goat MMC, GL and GMCP distribution carried out so far (Chapter 4). This study, along with preliminary immunohistochemical findings outlined in Chapter 3, localised GMCP to the majority of MMC and GL in the gastro-intestinal mucosa and also demonstrated its presence in some mast cells present in other peripheral tissues. It was found that significant numbers of toluidine blue staining cells

in certain tissue sites did not contain GMCP. This indicated that, as in sheep, phenotypic heterogeneity occurs in goat mast cells on the basis of their protease content. The presence of a putative goat mast cell tryptase enzyme that appears to be antigenically distinct from GMCP was also noted. This enzyme was detected, using anti-human tryptase antibodies, in a significant proportion of MMC and GL in the gastro-intestinal tissues. Previous observations have identified a similar distribution for a putative mast cell tryptase enzyme in sheep. This protease requires further characterisation, since it is possible that it could be an important enzyme in goat intestinal tissues, playing a significant, although, as yet unspecified, role following MMC activation.

Molecular characterisation studies carried out on cDNA isolated from goat BMMC (Chapter 5) further demonstrated the similarity between GMCP and SMCP at the DNA level whilst cDNAs encoding novel proteases in both the sheep and goat BMMC were also found. These enzymes, designated as GMCP II and SMCP II, have yet to be detected as distinct proteins in mast cell populations either *in vivo* or *in vitro*. However, given their relatively poor identity with GMCP I and SMCP I in terms of their predicted amino acid sequences (50-60%), it is possible that they represent antigenically distinct proteins which were not recognised by the antibodies used in this study. It would therefore be interesting to find out whether these proteases are also expressed, and if so, in which cell populations (BMMC and/or MMC) and in what quantities. Meanwhile, studies involving the sequencing of the 3' end of GMCP I also require completion to determine whether the dual specific properties shown by the isolated enzyme (Chapter 3) are dependent on the presence of charged acidic residues at positions 189 and/or 226 of the primary amino acid sequence.

Apart from GMCP I, dual-specificity has now been noted in a number of other serine proteases including cathepsin G from human neutrophils (Hof *et al.*,

1996), SMCP I (Pemberton *et al.*, 1997a) and, bovine duodenase (Zamolodchikova, 1995a). Further studies examining the *in vitro* and *in vivo* properties of dual-specific enzymes are therefore necessary to determine the physiological relevance of their partial trypsin-like activity. These studies will be aided by the determination of primary amino acid sequences for both GMCP and SMCP (Chapter 5; S. Macaleese, unpublished observations) which will enable more detailed assessments to be made on the spatial arrangement of residues present within the enzymes' active sites. Such information will hopefully lead to to a greater appreciation of the structural requirements for potential ruminant mast cell protease substrates and inhibitors present *in vivo*.

Seven days after equivalent secondary challenges with *T.circumcincta* it was found that there was 100-1000 times less GMCP compared to SMCP present in goat and sheep abomasal tissues respectively (Chapter 6). This was coupled with significant increases in the tissue protease content of trickle-challenged lambs compared to primary challenged lambs, which were not detected similarly challenged goats. In addition, the post mortem distribution and numbers of larvae present at each stage of development within gastro-intestinal tissues of the two species were also found to be significantly different. These findings suggest that lambs, in contrast to goat yearlings or kids, are able to regulate their worm burdens either by reducing the establishment of incoming larvae and/or by increasing the expulsion of already established adult worms. Similar findings regarding sheep immune responses were made by Huntley *et al.* (1992b) where it was found that gastro-intestinal mastocytosis and elevated tissue levels of SMCP were associated with resistance in sheep trickle-challenged with *H. contortus*. These responses involved the rapid expulsion (RE) of both incoming and established larvae in a process which, on the basis of current evidence, appears to be much less prevalent in the goat. Given the significant differences found in tissue protease levels found between the species, this suggests

that mast cell responses and tissue protease levels may play a critical role in mediating RE-type responses seen in trickle-challenged lambs as they develop acquired immunity to nematodes. However, the current study also highlighted other possible causes for the increased numbers of retained larvae in the goat tissues, including the presence of significantly higher abomasal pHs in the goats compared to the sheep. Further experiments are therefore required to examine the causes underlying alterations in goat abomasal pH values following infection, as well as their potential effects on the development and fecundity of intestinal worms.

The precise mechanisms by which dual-specific ruminant MMC-derived proteases may influence the development of gastro-intestinal immunity have yet to be established, although *ex vivo* evidence in a rat intestinal perfusion model indicates that RMCP II plays a significant role in causing rapid increases in intestinal-epithelial permeability following MMC activation (Scudamore *et al.* 1995 a and b). The absence of significant histological damage to the perfused mucosa following administration of RMCP-II also suggests that these alterations in permeability occur through the selective cleavage of protein substrates present within paracellular junctional complexes (Scudamore 1995 b). Possible targets for this mechanism may include the rat equivalent of a chicken zonula occludens protein, occludin, which from its predicted amino acid sequence, has several chymotrypsin-sensitive sites in its extracellular domains. Perfusion techniques have not been applied to ruminant studies, although isolation and purification of both GMCP and SMCP (as well as bovine duodenase) should make further investigations into activities of ruminant mast cell proteases against putative junctional protein complex substrates *in vitro* more feasible. Furthermore, studies *in vivo* could also be conducted to examine the relationships between intestinal tissue protease concentrations and the comparative permeabilities of parasitised goat and sheep mucosal epithelia to macromolecules including immunoglobulins, serum albumin and parasite antigens during and after challenge with

nematode larvae. Such experiments could exploit the the surgically modified intestinal loop technique described recently in sheep (Pernthaner *et al.* 1996), which would allow the local administration of purified mast cell proteases as well as the serial sampling of intestinal contents and mucus. Recent gene knockout experiments producing transgenic mice lacking the gene for the murine intestinal MMC protease MMCP-1 may also provide answers regarding the *in vivo* role of mast cell proteases in murine models of intestinal nematode infections (H. R. P. Miller, personal communication) although, due to technical difficulties, this approach is unlikely to be applied directly to ruminant studies in the near future.

The ELISA for GMCP could not detect protease in serum samples from parasitised goats or sheep or abomasal gastric lymph collected from ADCM challenged does (data not shown). These results may simply have been due to protease concentrations in the samples being below the sensitivity threshold of the assay, although another possible reason is the presence of serine protease inhibitors such as alpha-1-proteinase inhibitor and alpha-2-macroglobulin in the serum and lymph. Evidence for the latter was suggested by the finding that pre-incubation of detectable concentrations of purified GMCP or SMCP with goat and sheep serum respectively caused complete inhibition of the enzymes' catalytic activity against BLT (Chapter 3). In the sheep, these inhibitors have been shown to bind irreversibly to SMCP in serum, making the resulting complexes non-reactive with anti-SMCP antibody (A. D. Pemberton and J.F. Huntley personal communication). Therefore, it is possible that substantial amounts of GMCP released from MMC may be present in this undetectable form. If so, the differences observed in tissue concentrations of GMCP and SMCP in parasitised animals may be due to variations in the kinetics of protease storage and release, and not due to different levels of synthesis in the two species. Further studies to investigate GMCP synthesis and release kinetics as well as the potential interactions between GMCP and serum or lymph derived inhibitors are

therefore warranted. In the case of the former, experiments could involve comparative ELISA analysis of tissue samples taken at serial time points following primary and secondary nematode challenges or they could make use of the availability of cDNA probes for GMCP I and SMCP I (Chapter 5) to analyse comparative protease mRNA levels in the same samples by Northern blotting or semi-quantative RT-PCR.

The study described in Chapter 6 also provided the first comparative data on serum IgE levels present in these two species. Unfortunately, due to technical limitations, the findings were restricted to total serum IgE levels and not parasite-specific IgE. However, both species demonstrated elevations in their total serum IgE levels following a large primary or secondary challenge with 50,000 *T. circumcincta* L_3. IgE levels in yearling goats during trickle challenge were also found to be marginally higher than in the equivalently challenged lambs. Such findings may have been due to age differences between the yearling goats and the six month old lambs, or from more continuous or longer antigenic stimulation due to comparatively higher intestinal worm burdens present in the goats. However, until techniques have been developed to measure parasite-specific IgE antibody selectively, interpretation of this data will have to remain largely speculative.

Histochemical, immunohistochemical and ultrastructural analyses of goat GL both *in situ* and after isolation suggested that they are related to MMC on the basis of their GMCP content, putative tryptase content and residual granule morphology. (Chapter, 3; Chapter 4 and Chapter 8). In contrast, findings by Konno *et al.* (1994), demonstrated that that γδ T cell receptors are present on the surface of caprine GL and that there are differences in the glycoaminoglycan content between caprine GL and MMC. On the basis of these findings, they concluded that goat GL are not derived from MMC but represent a γδ T cell subset of caprine granulated intra-epithelial lymphocytes (GIEL). However, the surface receptor and biochemical characteristics of goat GIEL have not been fully investigated, whilst the presence of

GMCP in goat MMC/GL cells suggests that they are not derived from GIEL since studies in other species have demonstrated antigenically distinct proteases in this latter cell population (Huntley, McGorum, Newlands and Miller, 1984b). In the light of these conflicting proposals for the origin of caprine GL, further investigations to characterise completely the surface receptor and biochemical properties of caprine MMC, GL and/or GIEL are now required. Such studies could be facilitated by the application of the techniques described for the isolation of goat MMC and GL (Chapter 8) although, due to a current lack of goat-specific reagents, surface receptor characterisation studies will probably have to rely on the cross-reactivity of heterologous ovine and bovine monoclonal antibodies. (Konno *et al.* , 1994; Navarro *et al.*, 1995). However, the results of the current studies in the goat do appear to support similar findings in sheep, where intestinal GL have been convincingly demonstrated as an end-stage maturation of MMC (Huntley *et al.* 1984a).

The consistent finding that a much larger proportion of the total mast cell population present in parasitised goat intestines were present as GL despite the continued presence of significantly higher total worm burdens (Huntley *et al.*, 1995; Chapter 6) is intriguing, particularly as the presence of these cells in the sheep is strongly associated with the ability to rapidly expel worms (O'Sullivan & Donald 1973; Stankiewicz *et al.*, 1995). Whether the increased proportions of GL present in parasitised goat tissues are due to chronic antigenic stimulation of mast cells by the continual presence of relatively higher levels of nematode antigens, or whether it is due to the failure or absence of some negative feedback mechanism which limits sheep MMC activation and subsequent GL formation, is currently unknown. The possibility of functional differences in GL activity between the two species also requires investigation in experiments similar to the functional studies on goat BMMC described in Chapter 8. These experiments could include measuring the functional activity of sheep versus goat GL *in vitro*, through the release of mediators and/or

cytokines, following activation with secretogogues or IgE/antigen complexes. However, such studies require techniques which enable the *in vitro* study of pure populations of caprine and ovine GL and, as yet, these have not been successfully developed. Crude attempts were made to induce GL formation *in vitro* from caprine and ovine BMMC by continual stimulation with secretogogues and worm antigens. These were unsuccessful despite the demonstration of BMMC activation through the release of mediators (data not shown). This suggests that other components are required to induce the cell-type switching *in vivo*. These may include factors released by other cell types in the intestinal micro-environment including, T cells, B cells and epithelial or stromal cells. Future studies in this area might therefore consider co-culturing BMMC with other cell types at the time of antigen-mediated cell activation to see whether this proves more successful.

The present study demonstrated the heterologous activity of ovine rOvIL-3 and rOvSCF in generating goat BMMC (Chapter 7), indicating that these cytokines are important in the differentiation and maintenance of mature MMC populations within small ruminant intestinal-tissues *in vivo*. The current availability of rOvIL-3 and rOvSCF cDNA probes (McInnes *et al.*, 1993; C. McInnes unpublished observations) will enable further studies investigating the comparative levels of cytokine expression within goat and sheep intestinal tissues. Results from such experiments may provide further insights into local factors controlling the phenotype and activity of the increased mast cell and GL populations observed in the gastro-intestinal tissues of both species following challenge (Chapter 3 and Chapter 6).

In terms of *in vitro* studies into ruminant intestinal mast cell function, the most convenient cell source would be obtained by the production of transformed cell lines exhibiting the phenotypic characteristics of isolated ruminant MMC. However, this has yet to be achieved in goats and sheep. Attempts at producing immortalized ovine BMMC through retroviral-mediated transfection of oncogene DNA have so far

proved unsuccessful (Huntley, 1991). Until this can be realised, cytokine generated BMMC cultures remain the most convenient, and consistent, method for analysing the functional activity of ruminant mast cells *in vitro*. This was highlighted by the studies in Chapter 8 where goat jejunal MMC and GL were found to be functionally inactive following isolation, possibly due to damage incurred during the purification process. As a result, although structural and biochemical characterisation studies demonstrated qualitative similarities in mediator content between isolated MMC and BMMC it was impossible to characterise fully the BMMC as being phenotypically MMC-like on the basis of their functional activity. Nevertheless, secretogogue release studies carried out on caprine BMMC demonstrated similarities with MMC-like functional characteristics as demonstrated in other species and, on this basis, activation studies involving nematode antigens were carried out.

In the nematode antigen release studies (Chapter 8), the most significant findings were that mediator release from both caprine and ovine BMMC is possibly induced from non-sensitised cells by worm antigens alone and that this response may be enhanced by pre-sensitising with serum or lymph containing homocytotropic antibodies. Binding of these antibodies to the surface of both MMC and BMMC could also be demonstrated by immunofluoresence. Unfortunately, confirmation that this Ig was IgE was not possible due to the lack of antibodies recognising ovine or caprine native IgE. It is therefore possible that the enhanced release observed was mediated by an antibody isotype other than IgE. Eurther studies utilising monoclonal antibodies to other ovine antibodies such as IgG_1 and IgM could be used to rule out the binding of these isotypes, but due to time constraints, this was not possible. However, the finding that maximal cell activation followed cell sensitisation with native serum and lymph samples from immune animals containing detectable levels of total IgE suggests that antigen-mediated cross-linking of IgE bound to FcεRI receptors may be occurring. Mediator release achieved by sensitised BMMC activated

with nematode antigen preparations was usually found to be approximately half that obtained when using optimal concentrations of calcium ionophore A21387 during the characterisation studies. This could indicate that different activation mechanisms are operating in each case although, again due to time constraints and limited reagent stocks, comprehensive dose and time response studies for the nematode antigen release experiments were not carried out. Thus, although the BMMC were usually harvested at time points when they contained optimal amounts of mediators (Chapter 7), the highly variable percentage release obtained in the presence of the different nematode antigen preparations may have resulted from sub-optimal antigen concentrations and/or incubation periods in the release protocol. Future work in this area should therefore be aimed at standardising and extending these studies through a comprehesive series of time and dose response experiments for each individual antigen preparation.

The fact that BMMC sensitised with heat treated serum and lymph also released significant quantities of their mediators in response to stimulation with nematode antigens was also highly important as this may indicate the presence of additional release mechanism(s) which induce mast cell degranulation despite the apparent absence of surface bound immunoglobulins. Such antibody-independent mechanisms may be important *in vivo* for stimulating rapid MMC-mediated responses prior to the onset of the classical cell-mediated and humoral immune responses following exposure and APC processing of parasite antigens. As outlined in the general introduction, activated MMC have been shown to release a potent variety of mediators and cytokines that can influence Th and effector cell responses as well as humoral responses through Ig production and isotype switching. Therefore, it is possible the 'sentinel-like' anatomical distribution of MMC in the gastro-intestinal mucosa coupled with this rapid antibody-independent response mechanism, plays an important role in initiating and co-ordinating other aspects of the host's immune

response as soon as mucosal exposure to nematodes occurs. However, the antigens, receptors and signal transduction pathways involved in mediating this response are all currently unknown and require further study. Future experiments should also involve controls to test that the response is not due to endotoxin-mediated cytotoxicity of the antigen preparations, whilst efforts should also be made to ensure that residual IgE antibody, despite heat treatment and negative surface immunofluorescence, was not responsible either. In the case of the latter, pilot studies incorporating non-sensitised BMMC incubated with *T.c.* L3 WWA alone demonstrated similar release responses to BMMC incubated with heat inactivated serum (data not shown). This provided further evidence for a possible non-antibody mediated release mechanism and also indicated that release due to residual antibody activity in the heat treated serum control samples was likely to be negligible. The current release experiment also calculated mediator release as a percentage of the total BMMC content present in cell lysate preparations (Chapter 2.) however, future studies would also benefit from a more appropriate positive control involving the use of mast cells that have been triggered by surface cross-linking of IgE. This could possibly be achieved by incubating sensitised BMMC with an optimal concentration of anti-ovine light chain antibody.

The main aim of the current study was to assess the responses of caprine mast cells to stimulation with different nematode antigens and as a result, the granule associated mediators measured were chosen for their specificity (GMCP), relative abundance (β-hexosaminidase) and convenience (β-hexosaminidase and aryl-sulphatase). However there are other granule-associated mediators that have yet to be examined in these cell populations, including most notably histamine, serotonin (5-HT) and PGD_2 which have all previously been demonstrated in ovine BMMC and MMC (Huntley, 1991). In addition, this release protocol could also be modified to investigate the comparative production of other mediators, including cytokines, which

may be involved in the regulation of both mast cell-mediated and other cellular and humoral responses to nematodes (see general introduction). This is becoming increasingly possible with the development of cDNA probes and monoclonal antibodies to a variety of cloned and expressed ruminant cytokines (reviewed Wood and Seow, 1996). The expression of different recombinant ruminant cytokines may also allow further studies on the *in vitro* generation of BMMC enabling production of cell populations that more closely resemble isolated MMC in terms of their granule morphology and quantitative mediator content (Chapter 8).

In summary, the findings presented here have confirmed that significant differences exist in terms of the mediator content of goat and sheep intestinal mucosal MMC *in vivo*. However *in vitro* studies have also demonstrated that mast cell populations from both species can be activated to release their granule-associated mediators following exposure to secretogogues and nematode antigen preparation. It is my contention therefore, that MMC are intimately involved in intestinal hypersensitivity reactions which help to reduce nematode burdens in immune animals by preventing larval establishment and increasing the expulsion of adult worms. Key to this response is the antigen-mediated release of of neutral granule proteases from MMC which, although it has yet to be demonstrated conclusively in ruminants, may potentiate nematode expulsion by selectively altering mucosal epithelial permeability to the pathotopic transfer of worm antigens and immunoglobulins. In this respect, the profound lack of GMCP found in parasitised goat gastro-intestinal tissues could contribute significantly to their continued susceptibility to nematode infections even as adult animals.

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APPENDIX A

BUFFERS AND FIXATIVES

Phosphate buffered saline (x10 stock solution)

80g NaCl
2g KCl
11.5g Na_2HPO_4
2g KH_2PO_4

Dissolve in 1 litre dd H_2O to provide a x10 stock solution. Dilute 1:10 in dd H_2O and adjust pH to 7.2-7.3 prior to use

1M Tris/HCl stock solution

Dissolve 121.12g Tris (Sigma Cat. No. T-8524) in 900mls dd H_2O, pH to 7.5 by dropwise addition of concentrated HCl. Make up to 1 litre with dd H_2O. 0.01% NaN_3 may be added as a preservative.

20mM working Tris buffer made by mixing 20ml of the above with 980mls ddH_2O

GMCP purification and characterisation buffers

Tissue homogenate extraction buffers:

GMCP purification

homogenising buffer: 20mM Tris/HCl pH 7.8
tissue extraction buffer: 20mM Tris/HCl pH 7.8 + 1.0M NaCl

GMCP/SMCP ELISA extraction buffer for tissues and cell pellets

20mM Tris/HCl pH 7.8 + 1.5M NaCl + 0.01% NaN_3

FPLC buffers:

CM-Sepharose column
bufferA: 20mM Tris HCl pH 7.8
bufferB: 20 mM Tris HCl pH 7.8 + 1.0M NaCl

Mono S column
bufferA: 20mM Tris HCl pH 7.8
bufferB: 20 mM Tris HCl pH 7.8 + 1.0M NaCl

Synthetic substrates tested against GMCP:

Carboxybenzoyl-L-Tyrosine nitrophenol ester (Sigma Cat. No. C-3637)
Carboxybenzoyl-L-Tryptophan nitrophenol ester (Sigma Cat. No. C-5502)
Carboxybenzoyl-L- Phenylalanine nitrophenol ester (Sigma Cat. No. C-4502)
Carboxybenzoyl-L-Alanine nitrophenol ester (Sigma Cat. No. C-4635)
Carboxybenzoyl-L-Argnine nitroanilide (Sigma Cat. No. C-4893)
Carboxybenzoyl-L-Tyrosine nitroanilide (Sigma Cat No. B-6760)

all were dissolved in 100% dimethyl-sulphoxide (Sigma Cat. No. D-8386) to produce 5mM stock solutions

Carboxybenzoyl-L-Lysine thiobenzyl ester (Sigma Cat. No. C-3647) was dissolved in DMSO to produce a 10mM solution which was diluted 1: 1 with a 10mM solution of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent) (Sigma Cat. No. D 8130)

Working buffer for substrate assays:

0.1M Tris/HCl pH 7.8
(Reactions stopped by adding 100µl 100% methanol)

SDS-PAGE buffers

SDS gels:

12% resolving gels (10mls)

2.5mls 1M Tris/HCl pH 8.8 (resolving buffer)
4mls 30% acrylamide, 0.8% bis-acrylamide in ddH_2O (Easigel, Scotlab, Glasgow)
2.8mls ddH_2O
0.5mls 10% sodium dodecyl sulphate (SDS) in ddH_2O
10 µl N,N,N',N'-tetramethylethylendiamine (TEMED) (Biorad systems Cat. No. 161-0800)
100µl 10% ammonium persulfate in ddH_2O

Azocasein gels made up as above but with 20mg azocasein (Sigma Cat. No. A 2765) added prior to casting.

7.5% resolving gels (10mls)

2.5mls 1M Tris/HCl pH 8.8 (resolving buffer)
2.5mls 30% acrylamide, 0.8% bis-acrylamide in ddH_2O
4.4mls ddH_2O
0.5mls 20% SDS in ddH_2O

10 µl TEMED
100µl 10% ammonium persulfate in ddH_2O

4% stacking gels (5.4mls)

1.4mls1M Tris/HCl pH 8.8 (stacking buffer)
0.7mls 30% acrylamide, 0.8% bis-acrylamide in ddH_2O
3.09mls ddH_2O
0.14mls 20% SDS in ddH_2O
10 µl TEMED
100µl 10% ammonium persulfate in ddH_2O

Sample buffer:

Reducing buffer (10mls)

2.5 mls stacking buffer
2.3 mls ddH_2O
2.0 mls glycerol
2.0mls 20% SDS in ddH_2O
1 ml β-mercaptoethanol (Sigma Cat. No. M 6250)
0.2 mls 0.1% bromophenol blue (sodium salt) (Sigma Cat. No.B 7021)

Non-reducing buffer made up as above but without the addition of β-mercaptoethanol

Laemmli Tris/glycine running (tank) buffer pH 8.3 (x 5 stock solution, dilute in ddH_2O to provide 1 x working solution):

37.85g Tris
180.12g glycine
12.5g SDS
2.5 litres ddH_2O

Silver stain development solutions:

Fixing solutions
a) 50 mls methanol, 10 mls glacial acetic acid made up to 100mls with ddH_2O
b) 5mls methanol, 7mls glacial acetic acid made up to 100mls with ddH_2O

Reducing solution
1mg dithiothreitol (Sigma Cat. No. D-5545) in 20mls ddH_2O

Staining solution
100mg $AgNO_3$ in 100mls ddH_2O

Developing solution
3g $NaCO_3$ and 50µl 37% paraformaldehyde in 100mls ddH_2O

Stop solution
67.6 g citric acid dissolved in 100mls ddH_2O

Coomassie stain for azocasein substrate gels and amino acid sequence blots:

Stain
5g Coomassie brilliant blue R (Sigma Cat. No. B0149)
450mls ethanol
100mls glacial acetic acid

add dye to the ethanol /acetic acid, leave overnight at room temperature, filter (Whatman's No.1) and add 450mls ddH_2O

Destainer
250mls ethanol, 750mls glacial acetic acid make up to 2 litres with ddH_2O

Western blotting and dot blotting buffers

Western blotting

Transfer buffers:

Anode buffer 1:
0.3M Tris, 20% methanol; pH 10.4
(150mls 1M Tris + 100mls methanol + 250mls ddH_2O)

Anode buffer 2:
25mM Tris, 20% methanol; pH 10.4
(12.5mls 1M Tris + 100mls methanol + 387.5mls ddH_2O)

Cathode buffer:
25mM Tris/40mM, 40mM 6-amino-n-caproic acid (mwt. 131.2; Sigma Cat. No. A 2504), 20% methanol pH 9.4
(12.5mls 1M Tris + 40 mls 0.5M 6-amino-n-caproic acid + 100mls methanol + 347.5mls ddH_2O)

Western and dot blotting

Blocking , washing and antibody diluent buffer:

PBS + 0.02% Tween 80 (polyoxyethylenesorbitan-monooleate, Sigma Cat No. P-1754) + 0.5M NaCl

Immunoaffinity column buffers

Protein A, Protein G and Polyclonal anti-GMCP columns:

bufferA: PBS + 0.5M NaCl
bufferB (elution buffer): 0.1M Citrate + 0.5M NaCl pH 2.2
Neutralisation buffer: 1M Tris pH 10.8

Antibody production

Adjuvants

Primary dose: Freund's complete adjuvant (Sigma Cat. No. F 5881) (polyclonal antibody) or QuilA (Superfos Biosector Batch No. C77-67) (monoclonal antibodies)

Booster doses: Freund's incomplete adjuvant (Sigma Cat. No.F 5506) (polyclonal antibody) or QuilA (monoclonal antibodies)

Monoclonal antibodies

NS0 murine myeloma cell culture medium

500mls RPMI 1640 (Gibco BRL, Life technologies Ltd. Cat. No. 0430514OD) + 10ml penicillin/streptomycin solution (P/S) (10000 units penicillin and 10000 µg streptomycin per ml.

Hybridoma cell culture media

Plating medium containing hypoxanthine, thymidine and aminopterin (HAT): 500mls RPMI 1640 + 10mls P/S + 50-75 mls heat inactivated FCS (Advanced Protein Products Cat. No. AS 302 50) + 0.075g oxaloacetic acid (OAA) (Sigma Cat. No. O 9405) + 0.025g pyruvate (PYR) (Sigma Cat. No. P 3662) + 0.0041g insulin (INS) (24 $I.U.mg^{-1}$; Sigma Cat. No. I 4011) + 10ml x 50 HAT (Sigma Cat. No. H 0262) + 5mls 1M 3-[N-morpholino] propanesulphonic acid (MOPs) (Sigma Cat. No. M 5162)

* FCS heat inactivated at 55 ^{o}C for 2 hours prior to use in all tissue culture media

Feeding medium (for use after the first sub-cloning of the hybridoma cells)
Same as above but without the addition of HAT

Histochemistry & immunohistochemistry

Fixatives

4% Paraformaldehyde/PBS

4g paraformaldehyde dissolved in 100mls PBS. Heat in a fume cupboard to dissolve the paraformaldehyde. Cool to room temperature for tissue fixation or 40^{o}C for cytosmear fixation.

Modified Bouin's Fixative

Add 10mls of 40% paraformaldehyde and 5mls of glacial acetic acid to190mls saturated aqueous picric acid.

Graded alcohol series for dewaxing, rehydrating and dehydrating slides: (all steps carried out with fresh solutions and require regular agitation)

Dewaxing:	2 x 5 minutes in 100% xylene
Rehydrating:	3 minutes in equal parts 100% ethanol (74OP) and 100% xylene
	3 minutes in 74OP
	3 minutes in equal parts 74OP and ddH_2O
	5 minutes in ddH_2O
Dehydrating:	5 minutes in 89% ethanol (64OP)
	3 minutes in equal parts 74OP and ddH_2O
	3 minutes in 74OP
	3 minutes in equal parts 74OP and 100% xylene
Clearing:	2 x 5 minutes in 100% xylene

Histochemical stains

Haematoxylin

Ehrlich's haematoxylin original formula, Gurr® microscopy materials, (BDH, Cat No. 350172Q)

Toluidine blue (Enerback 1966a)

0.5% toluidine blue (Sigma Cat. No.T 3260) in 0.5N HCl pH 0.5 (Check pH and filter (Whatman's No. 1) before use)

Carbol chromotrope (Lendrum 1944)

5g chromotrope (Chromotrope 2R, Gurr® microscopy materials, (BDH, Cat No. 34020HP)
10g Phenol
dissolve in 1litre ddH_2O.

Leishman's stain

Leishman's staining solution (eosin methylene blue compound 0.2% w/v in methanol, Gurr® microscopy materials, (BDH, Cat No. 35022)

Carboxybenzoyl-l-lysine thiobenzyl ester (BLT) stain

incubation solution for 4% PF/PBS fixed cytosmears or tissues:
50ml 0.2M Tris pH 8.2 (pH with concentrated HCl)
10 mg Fast Blue BB (Sigma Cat. No. F 0250) - dissolve thoroughly
4.2mg Carboxybenzoyl-L-Lysine thiobenzyl ester (Sigma Cat. No. C-3647)

Immunohistochemical solutions

Endogenous peroxidase blocking solution

methanol + 0.5% H_2O_2
(0.5mls H_2O_2 added to 98.5 mls methanol)

Blocking and antibody diluent solution

4% bovine serum albumin /PBS
(Dissolve 4g BSA in 100ml PBS)

Peripheral blood eosinophil staining

Carpentier's Stain

2% aqueous Eosin Y (Sigma Cat. No. E 6003), 0.5g in 25ml ddH_2O
37% formaldehyde (Sigma Cat. No. F 1635) saturated with calcium carbonate. Make up saturated formaldehyde by adding 0.5g $CaCO_3$ per 100ml formaldehyde. Carpentier's stain prepared from 2ml of 2% Eosin Y and 3ml of 37% formaldehyde added to 95ml ddH_2O.

Helminthological iodine

250g potassium iodide
50g resublimed iodine
dissolve in 500ml tap water

DNA sequencing

TBE buffer (x5 stock solution, dilute in ddH_2O before use)

272.5g Tris, 139g Boric acid and 100 mls 0.5M EDTA
dissolve in 5 litres ddH_2O

Gels

Formaldehyde and agarose gels for RNA and DNA detection respectively were all separated using Hoefer gel apparatus (Horizon 58 or 11-14; Hoefer Scientific) submerged in 1 x MOPs buffer (Sigma Cat. No. M-5162) (formaldehyde gels) or 1 x TBE Buffer (agarose gels).

Formaldehyde gels for RNA detection

0.4g agarose (Sigma Cat. No. A 9539)
4ml 10 x MOPs pH 7.0;
36ml RNase/DNase free ddH_2O

Melt in a microwave oven (600 Watts medium setting for 2 minutes) allow to cool to < 50°C. Add 2ml of 37% formaldehyde (37g formaldehyde in 100ml RNase/DNase free ddH_2O) before casting the gel

Agarose gels for DNA detection

Mix 0.8g agarose (Sigma Cat. No. A 9539) in 100mls TBE and add 80µl of 0.5mg/ml ethidium bromide solution. Heat in a microwave oven (600 W medium setting for 2 minutes) allow to cool to < 50°C before casting the gel.

Northern running buffer/Agarose sample buffer

50% glycerol
0.1 mg/ml bromophenol blue (Sigma Cat. No. B 5525)
in RNase/DNase free ddH_2O

PCR reactions
Primary PCR mixture

5 µl 10 x PCR buffer (Boerhinger Mannheim Cat. No. 1 647 679)
5µl primer 1 (10µM)
5µl primer 2 (10µM)
2µl reverse transcriptase reaction product
5µl nucleotide mixture (2µM dNTPs; Boerhinger Mannheim Cat. No.1 277 049)

26µl sterile ddH_2O
Reaction mixture overlayed with 40µl mineral oil to prevent evaporation
2µl DNA Taq polymerase (1 unit/µl) (Boerhinger Mannheim Cat. No. 1 647 679)

Secondary PCR mixture

5 µl 10 x PCR buffer
5µl primer 3 (10µM)
5µl primer 4 (10µM)
1µl primary PCR product
5µl 2µM dNTPs
26µl sterile ddH_2O
40µl mineral oil to prevent evaporation
2µl DNA Taq polymerase (1 unit/µl)

PCR amplification programme (used for all amplifications, contains a final 30 minute extension step to produce overlapping ends for ligation into PCR 2.1™ vector)

Temperature (°C)	Time (minutes)	Number of cycles	Process
94	10	1	cDNA strand separation
72	0.5	1	add Taq 'hot start'
55	0.5	1	binding
72	0.5	1	polymerising
94	0.5	30	cDNA strand separation
55	0.5	30	binding
72	1	30	polymerising
72	30	1	extension step
25	till samples removed		

Southern blotting and probe hybridisation hybridisation buffers

Denaturing solution
1.5M NaCl + 0.5N NaOH
(87g NaCl and 20g NaOH dissolved in 1litre ddH_2O)

Agarose gels containing PCR products for digoxigenin-labelled oligonucleotide probing washed for 15 minutes and 30 minutes in this solution

Neutralising solution
1.5M NaCl + 0.5M Tris + 0.001M EDTA pH 7.5
(87g NaCl, 60.56g Tris and 2mls of 0.5M EDTA dissolved in 1 litre ddH_2O. pH to 7.5 with concentrated HCl)

Denatured agarose gels neutralised for 15 minutes and 30 minutes prior to blotting onto Hybond N+ nylon membranes overnight

20 x SSC stock buffer: 3M NaCl + 0.3M Na-citrate pH 7.0
Dilute with ddH_2O to the required concentration (2 x SSC , 1 x SSC, 0.5 x SSC or 0.2 x SSC) and add 0.1% SDS (w/v)

Maleic acid buffer: 0.1M maleic acid + 0.15M NaCl pH 7.5
(11.61g maleic acid and 8.76g NaCl dissolved in 950ml ddH_2O. pH to 7.5 with 5M NaOH, make up to 1 litre with ddH_2O)

Maleic acid Tween wash buffer: Maleic acid buffer + 0.3% Tween 20 (Sigma Cat No. P -1379) (1.2mls Tween 20 in 400mls buffer)

Blocking buffer:
1% Blocking solution (Boerhinger Mannheim Cat. No. 1 096 176) in maleic acid buffer

Buffer 3: 100mM Tris + 100mM NaCl + 5mM $MgCl_2$ pH 9.5

Buffer for stripping blots: 0.2M NaOH + 0.1% SDS
(0.8g NaOH + 1ml 10% SDS in 100mls dH_2O)

Nutrient media for the selection and maintenance of pCR™2.1 plasmid transformed *E.coli* cells.

When handling *E.coli* cells and nutrient media, all materials used were initially flamed to prevent contamination.

Luria-Bertolani broth (LB) agar plates containing kanamycin and x-galactopyranoside (x-gal)

Heat 500mls LB agar (32g LB agar powder per litre of sterile ddH_2O; Sigma Cat. No. L 2897) for 10 minutes on low in a 600W microwave oven
Mix and simmer on low for a further 5 minutes
Incubate in a water bath at 55 ^{o}C for 55 minutes
Add 5 mls 5mg/ml kanamycin (Sigma Cat. No. K 0879) dissolved in sterile ddH_2O
Add 400µl 1mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (x-gal) (Sigma Cat. No. B9146)
pour into sterile petri dishes and flame prior to storing at 4 ^{o}C

LB containing universals for overnight growth of selected *E.coli* colonies

10mls LB media (25g LB broth base per litre of sterile ddH_2O Sigma Cat. No. L 3522) aliquoted into sterile, glass universals.
Add 10 µl of 1mg/ml ampicillin (Sigma Cat. No. A 2804) dissolved in sterile ddH_2O

MMC isolation, tissue culture and mast cell release media

HBSS/heparin tissue culture medium for bone marrow and MMC isolation

500ml Hank's balanced salt solution diluted from x10 stock solution in ddH_2O (Gibco BRL, Life technologies Ltd. Cat. No. 041-04020/1M).
10ml penicillin/streptomycin solution.
10 000 units heparin (tissue culture grade 1A, Sigma Cat. No. H3149)

IMDM/10% FCS tissue culture medium for BMMC

500ml Iscove's modification of Dulbecco's medium (Gibco BRL, Life technologies Ltd. Cat. No. 041-01980M)
50ml FCS (Advanced Protein Products Ltd. Cat. No. AS-302-50) (heat inactivated in a water bath at 56°C for 30 minutes)
10ml penicillin/streptomycin solution
50µl 2-ME (50mM 2-mercaptoethanol)

Earles + 2% FCS tissue culture medium for cell release studies

500ml Earles salt solution containing Ca^{2+} and Mg^{2+} without HCO_3 diluted from x10 stock solution in ddH_2O (Gibco BRL, Life technologies Ltd. Cat. No. 042-04050H)
10ml FCS

Discontinuous Percoll™ gradients for MMC isolation

Stock Isotonic Percoll (IP) solution

11.5 mls sterile Percoll™ (Pharmacia Bio-tech, Uppsala Cat. No. 17 0891 01) + 1ml x10 Hanks balanced salt solution without HCO_3 (Gibco BRL, Life technologies Ltd. Cat. No. 041-04020)

55% IP = 55mls IP + 45mls x1 HBSS without HCO_3

65% IP = 65mls IP + 35mls x 1HBSS without HCO_3

80% IP = 80mls IP + 20 mls x 1HBSS without HCO_3

Gradients made up with 2mls 80% IP overlayed with 4 mls 65% IP. Cells resuspended in 55% IP to give a volume of 2 mls per gradient containing a maximum of 2×10^7 cells per gradient

SMCP/GMCP ELISA buffers

Carbonate coating buffer

0.1M $NaHCO_3$ (0.84g $NaHCO_3$ in 100ml ddH_2O)
0.1M Na_2CO_3 (1.06g Na_2CO_3 in 100ml ddH_2O)

Titrate the 0.1M $NaHCO_3$ to pH 9.6 with the Na_2CO_3. Store at -20°C in 20ml aliquots until use.

Sample diluent and wash buffer

PBS + 0.05% Tween 20 (Polyoxyethylenesorbitan-monolaureate; Sigma Cat. No. P 1379) (1ml Tween 20 in 2 litres of PBS)

Antibody diluent buffer

PBS + 0.5% Tween 80 (Polyoxyethylenesorbitan-monooleate) (Sigma Cat. No. P 1754) + 0.5M NaCl = 5mls Tween 80 and 29.22g of NaCl in 1 litre of PBS

β-hexosaminidase assay buffers

Citrate buffer for substrate, pH 4.5

Solution A

300ml 1M NaOH (1M NaOH = 40g NaOH + 1litre ddH_2O)
31.5g Citric acid
Make up to 1 litre with ddH_2O

Solution B

0.1N HCL (1 N HCL = 89mls concentrated HCl + 1litre ddH_2O)

Working citrate buffer solution = 67.8ml of solution A + 32.2ml of solution B, titrated to pH 4.5

Substrate

p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Cat. No. N9376). 5mM substrate solution = 1.7mg substrate per ml of citrate buffer, pH4.5 (substrate dissolves slowly, leave on a rotary mixer for 60 minutes at room temperature before using.)

Glycine NaOH stop solution, pH 10.7

Solution A

7.5g glycine
5.8g NaCl

The above is made up to 500ml with ddH_2O

Solution B

0.2N NaOH

Working glycine NaOH solution = 52.8mls of solution A + 47.2mls of solution N, titrated to pH 10.7. (Chill the solution on ice prior to use)

Aryl-sulphatase assay buffers

Acetate buffer for substrate pH 5.7

0.2M sodium acetate (27.22g sodium acetate in 1 litre ddH_2O), titrated to pH 5.7 with concentrated HCl

Substrate

p-nitrocatechol sulphate (Sigma Cat. No. N-7251) 6.255mM substrate solution = 9.75mg substrate per 5ml acetate buffer, pH 5.7

Stop solution

5M NaOH (20g NaOH pellets dissolved in 100mls ddH_2O)

APPENDIX B

ADDITIONAL DATA

Group 1 (CI)	Abomasal worm burdens											
Yearlings	Early L4	Mid L4	Late L4	Total L4	Early L5 Male	Early L5 Female	Total Early L5	Adult Male	Adult Female	Total Adult	Total L5/Adult	Total Nos
Green1	19586	0	6049	25635	0	0	0	1729	1441	3170	3170	28801
Green2	28757	0	2026	30783	406	0	406	3646	5671	9317	9723	40501
Green3	18908	0	0	18908	549	275	824	4385	3289	7674	8498	27401
Green4	661	0	0	661	0	661	661	4621	5061	9683	10344	11001
Green5	3808	0	2539	6347	142	0	142	2539	5077	7617	7759	14101
Mean	14344	0	2123	16467	219	187	407	3384	4108	7492	7899	24361
SD	11771	0	2481	12720	248	290	344	1230	1737	2591	2831	11971
Kids												
White1	15687	0	4067	19754	0	0	0	2905	6391	9296	9296	29050
White2	2344	0	0	2344	0	0	0	10548	16408	26956	26956	29300
White3	23813	0	0	23813	0	0	0	4128	3810	7938	7938	31750
White4	11201	0	1461	12662	0	0	0	4870	6818	11688	11688	24350
White5	9872	0	0	9872	926	309	1234	9255	10489	19744	20978	30850
Mean	12583	0	1106	13689	185	62	247	6341	8783	15124	15371	29060
SD	7903	0	1772	8420	414	138	552	3356	4883	8043	8237	2858
Lambs												
Blue1	3829	0	0	3829	0	0	0	8463	7859	16322	16322	20150
Blue2	1632	0	0	1632	408	476	884	2244	2040	4284	5168	6800
Blue3	5358	0	0	5358	114	0	114	3420	2508	5928	6042	11400
Blue4	1078	0	0	1078	0	0	0	7392	6930	14322	14322	15444
Blue5	9683	0	0	9683	842	0	842	4631	5894	10525	11367	21050
Mean	4316	0	0	4316	273	95	368	5230	5046	10276	10644	14969
SD	3457	0	0	3457	359	213	455	2630	2629	5191	4936	5990

Group 2 (CC)	Abomasal worm burdens											
Yearlings	Early L4	Mid L4	Late L4	Total L4	Early L5 Male	Early L5 Female	Total Early L5	Adult Male	Adult Female	Total Adult	Total L5/Adult	Total Nos
Red1	4873	523	12008	17404	0	0	0	0	0	0	0	17401
Red2	981	219	9703	10903	0	0	0	0	0	0	0	10901
Red3	3366	282	5705	9353	0	0	0	0	0	0	0	9351
Mean	3074	341	9139	12553	0	0	0	0	0	0	0	12551
SD	1963	161	3189	4272	0	0	0	0	0	0	0	4271
Kids												
Yellow1	221	21733	0	21953	0	0	0	0	0	0	0	21951
Yellow2	511	9692	0	10203	0	0	0	0	0	0	0	10201
Yellow3	6877	12226	0	19103	0	0	0	0	0	0	0	19101
Yellow4	637	15266	0	15903	0	0	0	0	0	0	0	15901
Yellow5	5644	15259	0	20903	0	0	0	0	0	0	0	20901
Mean	2778	14835	0	17613	0	0	0	0	0	0	0	17611
SD	3213	4506	0	4736	0	0	0	0	0	0	0	4736
Lambs												
Orange1	1710	0	17290	19000	0	0	0	0	0	0	0	19000
Orange2	426	568	13206	14200	0	0	0	0	0	0	0	14200
Orange3	1107	1476	3567	6150	0	0	0	0	0	0	0	6150
Orange4	1482	1853	9016	12350	0	0	0	0	0	0	0	12350
Orange5	1068	2314	14418	17800	0	0	0	0	0	0	0	17800
Mean	1159	1242	11499	13900	0	0	0	0	0	0	0	13900
SEM	489	945	5340	5094	0	0	0	0	0	0	0	5094

Fig. 6.1a Individual post mortem abomasal worm burdens showing the numbers of larvae (L) present at each stage. CC = challenge control animals; CI = continuously infected animals

Group 1 (CC)		Cell counts					ELISA	
Yearlings	Abomasal pH	Tol Bl	Carb chrom	Total	anti-GMCP	Eosinophils	Abomasum	Jejunum
Green1	5.80	10.8	31	41.8	9	3.80	0.01	0.05
Green2	4.00	12	11.4	23.4	15	10.60	0.03	0.39
Green3	4.60	2.8	17.4	20.2	21	2.00	0.06	0.20
Green4	6.10	11.2	14.2	25.4	22.8	3.20	0.05	0.59
Green5	4.80	13.2	44	57.2	11.2	9.80	0.06	0.45
Mean	5.06	10.00	23.60	33.60	15.80	5.88	0.04	0.33
SD	0.87	4.13	13.66	15.61	6.00	4.01	0.02	0.21
Kids								
White1	7.10	6.8	9.4	16.2	10.8	5.20	0.04	0.23
White2	5.60	8.4	6.8	15.2	7	32.20	0.07	0.00
White3	4.20	8.4	1.8	10.2	5	18.80	0.06	0.57
White4	6.20	15.2	2.2	17.4	19.6	5.80	0.08	0.24
White5	5.20	5.8	6.8	12.6	8.6	4.60	0.06	2.49
Mean	5.66	8.92	5.40	14.32	10.20	13.32	0.06	0.71
SD	1.09	3.68	3.28	2.90	5.67	12.09	0.02	1.02
Lambs								
Blue1	3.70	5	0.2	5.2	10.6	4.20	80.85	3.65
Blue2	4.70	6.2	0.4	6.6	10.4	0.60	88.44	52.79
Blue3	2.20	16	0.2	16.2	0.2	2.20	116.38	16.09
Blue4	2.90	17.8	0.6	18.4	16	3.60	152.79	16.80
Blue5	4.50	7	2.8	9.8	3	5.00	31.98	3.26
Mean	3.60	10.40	0.84	11.24	8.04	3.12	94.09	18.52
SD	1.06	6.01	1.11	5.83	6.37	1.74	44.74	20.23

Group 2 (CI)		Cell counts					ELISA	
Yearlings	Abomasal pH	Tol Bl	Carb chrom	Total	anti-GMCP	Eosinophils	Abomasum	Jejunum
Red1	4.60	5	11.2	16.2	1.4	0.80	0.04	0.21
Red2	4.20	4.8	2.6	7.4	1.4	0.60	0.06	2.79
Red3	3.60	8.4	4	12.4	10.6	8.00	0.09	0.56
Mean	4.13	6.07	5.93	12.00	4.47	3.13	0.06	1.19
SD	0.50	2.02	4.61	4.41	5.31	4.22	0.02	1.40
Kids								
Yellow1	4.60	1.8	0	1.8	2.8	1.60	0.05	0.22
Yellow2	3.90	6.8	0.4	7.2	4.8	0.40	0.13	3.98
Yellow3	3.90	3.4	0	3.4	0	0.40	0.12	0.54
Yellow4	4.10	2.6	0.4	3	4.2	2.00	0.08	0.60
Yellow5	3.80	2.2	0	2.2	2.6	0.40	0.11	0.13
Mean	4.06	3.36	0.16	3.52	2.88	0.96	0.10	1.10
SD	0.32	2.01	0.22	2.15	1.86	0.78	0.03	1.63
Lambs								
Orange1	3.20	4.2	0	4.2	3.6	1.00	0.67	9.52
Orange2	3.40	1.2	0	1.2	0.8	0.80	0.15	0.14
Orange3	2.10	1.8	0.2	2	2.8	2.40	0.27	3.49
Orange4	1.60	3.2	0.2	3.4	4.6	0.20	0.15	7.75
Orange5	1.50	3.8	0.4	4.2	4.2	1.20	0.62	6.06
Mean	2.36	2.84	0.16	3.00	3.20	1.12	0.37	5.39
SD	0.89	1.29	0.17	1.35	1.50	0.81	0.25	3.68

Fig. 6.1b Individual post mortem abomasal pH, abomasal cell count and GMCP ELISA results. CC = challenge control animals
CI = continuously infected animals; Tol Bl = toluidene blue stained cells; carb chrom = carbol chromotrope stained cells
total = combined toluidene blue and carbol chromotrope results; anti-GMCP = cells numbers staining positive with polyclonal antibody to GMCP. Cell counts quoted as cells per 0.2mm^2, GMCP ELISA results as µg/g wet weight tissue.

BMMC Secretogogue release data

β-hexosaminidase

Ionophore	Experiment 1	Experiment 2	Experiment 3	Mean	SD
A21387 10^{-4}M	13.7	10.2	10.2	11.3	2.0
A21387 10^{-5}M	13.9	22.7	33.6	23.4	9.8
A21387 10^{-6}M	79.1	72.7	77.9	76.6	3.4
A21387 10^{-7}M	28.9	4.6	4.0	12.5	14.2
*Diluent + ethanol release	17.3	18.7	14.2		
Secretogogue					
Compound 48/80 (250 μg/ml)	2.6	4.2	0.03	2.3	2.1
Substance P 10^{-2}M	0.4	0	1.6	0.7	0.8
Substance P 10^{-4}M	0	0	0	0	0
**Diluent only	12.8	6.1	12.6	10.5	3.8

***Aryl-sulphatase

Secretogogue	Experiment 1	Experiment 2	Mean	SD
A21387 10^{-5}M	16	22.3	19.2	4.4
A21387 10^{-6}M	18.6	17.8	18.2	0.5
A21387 10^{-7}M	78.6	61.8	70.2	11.8
A21387 10^{-8}M	29.3	21.7	25.5	5.3
*Diluent + ethanol release	15.5	21.7		
Secretogogue				
Compound 48/80 (250 µg/ml)	0	0	0	0
Substance P 10^{-2}M	0	0	0	0
Substance P 10^{-4}M	0	0	0	0
**Diluent only	0	0.7	0.35	0.49

GMCP

Ionophore	Experiment 1	Experiment 2	Experiment 3	Mean	SD
A21387 10^{-4}M	7.5	11.9	16.9	12.1	4.7
A21387 10^{-5}M	23.8	31.8	17.3	24.3	7.2
A21387 10^{-6}M	44.9	40.6	32.4	39.3	6.3
A21387 10^{-7}M	26.4	23.5	15	21.7	5.9
*Diluent + ethanol release	14.8	13.4	15.5		
Secretogogue					
Compound 48/80 (250 µg/ml)	21.9	19.9	24.1	23.5	2.1
Substance P 10^{-2}M	8.7	0.2	1.6	3.5	4.5
Substance P 10^{-4}M	3.6	12.5	8.6	8.2	4.4
**Diluent only	6.4	2.4	15.5	8.1	6.7

Individual results for secretogogue release experiments described in Chapter 8. Values for each eperiment represent the mean percentage madiator release for duplicate samples.

* Cells incubated in diluent containing the equivalent concentration of ethanol required to dissolve 10^{-4}M A21387 (see chapter 2). A21387 results are corrected for this spontaneous release.

** Cells incubated in diluent only. Compound 48/80 and substanceP results are corrected for this spontaneous release.

*** Aryl-sulphatase data based on two experiments only due to the exceptionally low levels of this enzyme found in day 16 goat BMMC

BMMC worm antigen release data

Goat BMMC

i) % Beta-hexosaminidase release

Heat inactivated serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	12.54	14.59	0.00	9.04	7.90
T.circumcincta L3 WWA	16.04	0.00	3.48	6.51	8.44
T.circumcincta. L5 ES	7.26	10.33	0.02	5.87	5.29
T.circumcincta L5 WWA	0.00	0.02	0.00	0.01	0.01
H.contortus L5 ES	0.00	0.00	0.00	0.00	0.00
H.contortus L5 WWA	0.00	0.03	0.00	0.01	0.02

Lymph sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	9.04	19.06	8.62	12.24	5.91
T.circumcincta L3 WWA	15.80	23.74	10.44	13.66	6.69
T.circumcincta. L5 ES	12.11	8.08	11.50	10.56	2.17
T.circumcincta L5 WWA	0.72	0.41	0.50	0.57	0.16
H.contortus L5 ES	0.00	1.08	0.54	0.81	0.54
H.contortus L5 WWA	0.41	0.60	1.48	0.83	0.57

IgE positive serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	21.29	28.96	13.80	21.35	7.58
T.circumcincta L3 WWA	15.85	3.57	16.00	11.81	7.13
T.circumcincta. L5 ES	16.41	20.62	8.17	15.07	6.33
T.circumcincta L5 WWA	0.45	4.63	0.45	2.54	2.41
H.contortus L5 ES	0.00	1.05	0.00	0.52	0.61
H.contortus L5 WWA	0.00	6.91	2.18	3.03	3.53

Goat BMMC

ii) % GMCP release

Heat inactivated serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	9.96	10.25	0.00	6.74	5.83
T.circumcincta L3 WWA	22.24	62.77	29.08	38.03	21.70
T.circumcincta. L5 ES	6.02	4.72	0.00	3.58	3.17
T.circumcincta L5 WWA	0.00	0.71	0.00	0.36	0.41
H.contortus L5 ES	0.00	13.94	0.00	6.97	8.05
H.contortus L5 WWA	11.72	9.72	9.54	10.33	1.21

Lymph sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	0.00	17.55	16.83	11.46	9.93
T.circumcincta L3 WWA	62.71	73.45	44.57	60.24	14.60
T.circumcincta. L5 ES	1.98	6.54	2.47	3.66	2.51
T.circumcincta L5 WWA	0.00	0.71	0.00	0.36	0.41
H.contortus L5 ES	0.00	29.74	0.00	14.87	17.17
H.contortus L5 WWA	0.00	21.80	9.54	10.45	10.93

IgE positive serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	4.97	17.47	16.83	13.09	7.04
T.circumcincta L3 WWA	81.37	65.97	58.34	68.56	11.73
T.circumcincta. L5 ES	4.81	13.06	25.47	14.45	10.40
T.circumcincta L5 WWA	23.52	0.00	0.00	11.76	13.58
H.contortus L5 ES	0.00	36.69	0.00	18.34	21.18
H.contortus L5 WWA	26.43	26.21	0.00	17.55	15.20

Goat BMMC

iii)% Aryl-sulphatase release

Heat inactivated serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	17.72	8.16	18.40	14.76	5.73
T.circumcincta. L5 ES	3.38	9.71	17.40	10.16	7.02
T.circumcincta L5 WWA	3.65	1.00	0.00	2.32	1.89
H.contortus L5 ES	0.00	0.00	7.75	3.88	4.47
H.contortus L5 WWA	0.00	5.73	3.89	3.21	2.93

Lymph sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	19.32	17.03	15.46	17.27	1.94
T.circumcincta. L5 ES	6.41	47.14	16.02	23.19	21.29
T.circumcincta L5 WWA	6.41	38.78	0.00	22.60	20.79
H.contortus L5 ES	0.00	32.95	1.24	17.10	18.68
H.contortus L5 WWA	0.00	31.90	1.98	11.29	17.87

IgE positive serum sensitised

	expt 1	expt 2	expt 3	mean	SD
T.circumcincta L3 ES	41.80	11.92	17.51	23.74	15.89
T.circumcincta. L5 ES	42.69	58.49	13.14	38.11	23.02
T.circumcincta L5 WWA	28.98	49.06	0.00	39.02	24.66
H.contortus L5 ES	0.00	30.83	0.00	15.42	17.80
H.contortus L5 WWA	10.17	10.00	0.00	6.72	5.82

Sheep BMMC

i) % Beta-hexosaminidase release

Heat inactivated serum sensitised

	expt 1	expt 2	mean	SD
T.circumcincta L3 ES	7.87	3.43	5.65	3.15
T.circumcincta L3 WWA	6.55	1.38	3.97	3.65
T.circumcincta. L5 ES	3.28	2.49	2.88	0.56
T.circumcincta L5 WWA	0.00	0.00	0.00	0.00
H.contortus L5 ES	0.48	0.00	0.24	0.34
H.contortus L5 WWA	0.00	0.00	0.00	0.00

IgE positive serum sensitised

	expt 1	expt 2	mean	SD
T.circumcincta L3 ES	23.98	20.53	22.26	2.43
T.circumcincta L3 WWA	13.09	9.18	11.14	2.76
T.circumcincta. L5 ES	5.35	4.00	4.67	0.95
T.circumcincta L5 WWA	0.35	0.00	0.17	0.25
H.contortus L5 ES	0.52	0.00	0.26	0.37
H.contortus L5 WWA	0.00	1.38	0.69	0.97

ii) % GMCP release

Sheep BMMC

	Heat inactivated serum sensitised			
	expt 1	**expt 2**	**mean**	**SD**
T.circumcincta L3 ES	1.53	0.00	0.77	1.08
T.circumcincta L3 WWA	9.02	1.10	5.06	5.60
T.circumcincta. L5 ES	0.35	0.00	0.18	0.25
T.circumcincta L5 WWA	0.00	0.00	0.00	0.00
H.contortus L5 ES	0.00	0.00	0.00	0.00
H.contortus L5 WWA	0.00	0.00	0.00	0.00
	IgE positive serum sensitised			
	expt 1	**expt 2**	**mean**	**SD**
T.circumcincta L3 ES	30.44	14.48	22.46	11.28
T.circumcincta L3 WWA	20.54	80.12	50.33	42.13
T.circumcincta. L5 ES	6.06	0.00	3.03	4.29
T.circumcincta L5 WWA	0.00	0.00	0.00	0.00
H.contortus L5 ES	0.00	0.00	0.00	0.00
H.contortus L5 WWA	0.00	0.00	0.00	0.00

iii)% Aryl-sulphatase release

Sheep BMMC

	Heat inactivated serum sensitised			
	expt 1	**expt 2**	**mean**	**SD**
T.circumcincta L3 ES	15.10	9.04	12.07	4.29
T.circumcincta. L5 ES	11.16	9.95	10.55	0.86
T.circumcincta L5 WWA	0.39	0.00	0.19	0.28
H.contortus L5 ES	0.68	0.00	0.34	0.48
H.contortus L5 WWA	0.97	0.00	0.49	0.69
	IgE positive serum sensitised			
	expt 1	**expt 2**	**mean**	**SD**
T.circumcincta L3 ES	26.93	34.06	30.50	5.04
T.circumcincta. L5 ES	10.81	14.97	12.89	2.94
T.circumcincta L5 WWA	0.00	0.00	0.00	0.00
H.contortus L5 ES	0.00	0.00	0.00	0.00
H.contortus L5 WWA	0.00	0.00	0.00	0.00

Individual results for worm antigen release studies described in chapter 8. Values for each experiment represent the mean percentage release for duplicate samples. Results corrected for spontaneous release, release induced by sensitisation and enzyme activity in the antigen preparations.

APPENDIX C

PUBLICATIONS ARISING FROM THIS THESIS